AN ABSTRACT OF THE DISSERTATION OF

Jennifer Marie Duringer for the degree of Doctor of Philosophy in Toxicology presented on April 30, 2003.

Title: Metabolism of Toxic Plant Alkaloids in Livestock: Comparative Studies on the Hepatic Metabolism of Pyrrolizidine Alkaloids in Sheep and Cattle and of Ergot Alkaloids in an Endophyte-Resistant Mouse Model.

Abstract approved:

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A. Morrie Craig

The pyrrolizidine alkaloids (PAs) and ergot alkaloids are known natural toxicants found in livestock forage. These alkaloids contribute to large economic losses in livestock throughout the world. An understanding of the mechanisms of toxicity and development of better diagnostic tools for better management practices was investigated.

Variability exists in the toxicity of PAs in ruminants where cattle are more susceptible and sheep are more resistant. The mechanism of PA resistance in sheep has been attributed to hepatic metabolism or rumen microbial degradation of PAs to non-toxic moieties. The hepatic metabolism of the PA senecionine was investigated in cattle and sheep liver microsomes. The level of a toxic pyrrole metabolite 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine pyrrole (DHP) formed in cattle and sheep were similar. However, the level of a non-toxic *N*-oxide metabolite was greater in sheep than in cattle. Cytochrome P450 and flavin monooxygenases (FMOs) responsible for PA

oxidative metabolism were similar in both ruminant species. Therefore, hepatic metabolism of PAs is not solely responsible for resistance observed in sheep versus cattle.

Ergot alkaloids present in endophyte-infected plants also cause toxicity in livestock. HPLC is the typical method used to quantify ergot alkaloid content; however, it is costly and time-consuming. An enzyme-linked immunosorbent assay (ELISA) developed with lysergol as the hapten was evaluated to ascertain its feasibility as an analytical tool for the ergot alkaloids found in forage plants. The ELISA detected the presence of lysergic acid but was not a reliable assay for the ergopeptine alkaloids such as ergovaline.

The genetic divergence in mice previously selected into ergot alkaloid susceptible and resistant lines was studied after ten generations of relaxed selection. Physiologically no difference was seen between the susceptible and resistant line for average daily weight gain. However, hepatic metabolism of the ergot alkaloid ergotamine showed differences between genders and between animals on diets containing no ergot alkaloids or a high concentration of ergot alkaloids. Four major biotransformation products were identified as hydroxylated ergotamine isomers based on mass spectroscopic analysis.

Metabolism of Toxic Plant Alkaloids in Livestock: Comparative Studies on the Hepatic Metabolism of Pyrrolizidine Alkaloids in Sheep and Cattle and of Ergot Alkaloids in an Endophyte-Resistant Mouse Model

by

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APPROVED:

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to ay reader upon request.

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Jennifer Marie Duringer, Author

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I would like to thank all of my committee members for their input and support in helping me complete this work, as well as all the other students, technicians and professors who have lent an ear or a helping hand along the way. For you I am extremely grateful. I especially want to thank my major professor, Dr. Craig, for supporting me and for providing me with the adventure and opportunity to carry out this research. I would also like to thank Dr. Buhler and Marilyn Henderson for providing me the tools and training to complete the assays in this thesis.

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"In the end, we will protect only what we love. We will love only what we understand. We will understand only what we are taught."

~Senegalese naturalist Baba Dioum

CONTRIBUTION OF AUTHORS

Dr. Morrie Craig was involved in the design, analysis and writing of each manuscript. Dr. Donald Buhler assisted in the experimental design for Chapter 2 and provided laboratory space, equipment and supplies for the experiments performed in Chapters 2 and 4. Dr. Ronald Lewis and Dr. Tom Fleischmann were involved in the design and analysis for the experiments in Chapter 4.

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DEDICATION

I dedicate this manuscript to my husband, Timothy, and my parents Thomas Schnitzius, Sonny and Deborah Dawson, and Bob and Mary Duringer who have lovingly supported me through the duration of my education. Without you, this would have been a much tougher road to travel.

"Some people come into our lives and quickly go. Some people move our souls to dance. They awaken us to understanding with the passing whisper of their wisdom. They stay in our lives for a while, leave footprints on our hearts and we are never, ever the same."

~Anonymous

METABOLISM OF TOXIC PLANT ALKALOIDS IN LIVESTOCK: COMPARATIVE STUDIES ON THE HEPATIC METABOLISM OF PYRROLIZIDINE ALKALOIDS IN SHEEP AND CATTLE AND OF ERGOT ALKALOIDS IN AN ENDOPHYTE-RESISTANT MOUSE MODEL

Chapter 1

INTRODUCTION

Plants and animals are mutually dependent inhabitants of earth. The biosynthetic capacities of plants allow them to trap solar energy, synthesize organic compounds from carbon dioxide, and release oxygen as a waste product. Animals feed on plants as their source of nutrients, utilize oxygen from plants for respiration, and excrete carbon dioxide for use by plants. From an evolutionary and ecological standpoint, it is not in the best interest of either plants or animals for plants to be eaten to extinction by animals or for plants to be completely resistant to animal herbivory (Cheeke 1994). Since plants are immobile and unable to resist herbivory by moving, they must develop other means to avoid predation by herbivores. These means are primarily physical and chemical defenses. Physical defenses include thorns, spines, leaf hairs and highly lignified or silica-loaded tissue (Cheeke 1994). Chemical defenses that either reduce palatability or adversely affect the health of ingesting herbivores give the plant survival advantages in the field over other plant species (Craig 1995).

In response, animals have coevolved various responses to the adverse health consequences of plant toxins. Some species have developed a resistance to the toxicity caused by these plant compounds, while others have remained susceptible to toxicity. In ruminants, these responses include microbes in the digestive system which can biodegrade toxins prior to systemic absorption (Craig 1995, Rasmussen et al. 1993, Allison et al. 1992,

Craig et al. 1987, Jones and Megarrity 1986) and/or metabolism in the liver to compounds which are then excreted in urine or feces (Cheeke and Huan 1995, Cheeke 1994, Buhler et al. 1990). Ruminant animals include sheep, cattle, goats, deer and elk. They differ from other herbivores due to their segmented digestive system which centers on the first compartment, the rumen, as the primary site of digestion of plant material. Ruminants are optimally adapted to metabolize complex molecules, with the rumen acting as an anaerobic fermentation vat consisting of many species of bacteria, fungi and protozoa (Craig 1995, Orpin 1994, Hobson 1988, Orpin and Joblin 1988). These rumen organisms help digest plant material which in turn produces nutrients for the ruminant. Ruminal microbes vary in number and predominant species based on feed, antibiotic inhibition or promotion, and ecological competition. Microbes that can metabolize unique compounds in plants gain a survival and growth advantage when that substrate is present. Thus, certain plant constituents can promote the growth of the bacteria that will degrade them, which is critical when those constituents are toxic to the host animal (Craig 1995, Wachenheim et al. 1992b). The number of microbes and the rate of biodegradation by these microbes are important in limiting the systemic absorption of toxins. If the numbers of biodegrading microbes are low or their rate is too slow, the animal will be subjected to the toxic effects of the ingested compounds. In some cases, microorganisms work synergistically as a consortia to biodegrade complex molecules and plant material (Craig 1995, Craig and Blythe 1994, Allison and Rasmussen 1992).

Alternatively, ruminants may utilize enzymes and other components in the gastrointestinal system and liver to bioactivate and/or detoxify plant toxins. Rapid and extensive changes in biotranformational enzymes present in the gastrointestinal tract can play an important role in metabolism and absorption of xenobiotics (Klaassen 1996, Cheeke 1994, Smith 1992). The liver is strategically placed to prevent exposure to toxins for the rest of the body as it is first post-absorptive organ encountered by ingested toxins. Hepatic

enzymes including the Phase I enzymes cytochrome P450s, flavin-containing monooxygenases and carboxylesterases and the Phase II enzymes UDP-glucuronosyltransferases and glutathione-S-transferases are inducible which can trigger rapid bioactivation and detoxification once toxins are ingested (Klaassen 1996). There is a high degree of variability in these enzymes between tissues, individuals and species, allowing for a large range of susceptibility to ingested toxins.

Phase I reactions expose or introduce a functional group and usually result in only a small increase in hydrophilicity of xenobiotics (Klaassen 1996). The functional groups exposed or introduced during phase I biotransformation are often sites of Phase II metabolism. Among Phase I biotransforming enzymes, cytochrome P450s (P450s) rank first in catalytic versatility and the range of substrates they can activate or detoxify (Klaassen 1996). The highest concentration of P450 enzymes are found in liver endoplasmic reticulum (microsomes) but P450s are present in all tissues. Liver microsomal P450 enzymes play an important role in determining the intensity and duration of action of drugs. They can also catalyze the detoxification of xenobiotics, although metabolism by cytochrome P450s does not always lead to non-toxic products. Activation by cytochrome P450s can lead to increased toxicity or tumorigenicity of the parent molecule in some compounds. In a number of cases, the same P450 can catalyze both activation and detoxification reactions.

P450s are heme-containing proteins that require electrons from NADPH-cytochrome P450 reductase, NADPH and O_2 to carry out monooxygenation of a given substrate. The basic reaction carried out by cytochrome P450 oxygenation is as follows: substrate (R-H) + O_2 + NADPH + $H^+ \rightarrow$ product (ROH) + H_2O + $NADP^+$ (Figure 1.1). P450s are assigned to different families (CYP 1,2 etc.) based on a 40% amino acid sequence identity. Enzymes of the same subfamily (CYP2A, CYP2B etc.) are 40-55% identical, while

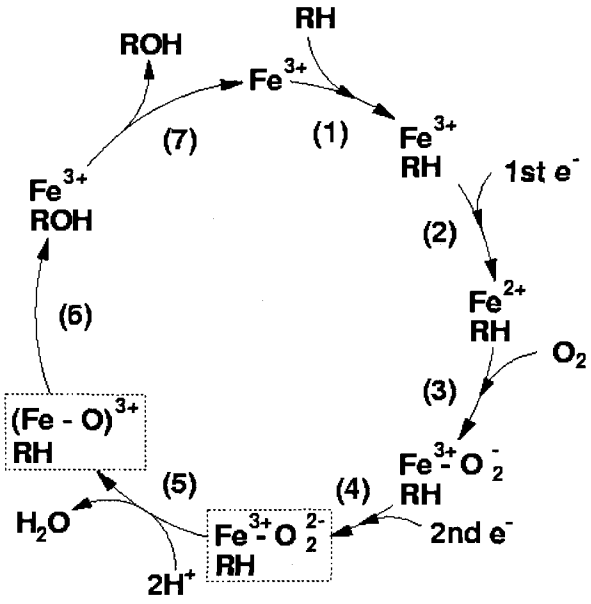


Figure 1.1 Catalytic cycle of cytochrome P450s. RH and ROH are the xenobiotic substrate and oxygenated product, respectively.

enzymes of the same species (CYP2A1, CYP2A2 etc.) are greater than 55% identical. In 1996, over 350 cytochrome P450 genes had been sequenced in a variety of organisms (Ioannides 1996). Many more are predicted to exist and it is suggested that we have just "hit the tip of the iceberg" in identifying the types and number of P450s.

P450s can be selectively inhibited by substrates to help determine which isozymes are responsible for metabolism of a compound of interest. For example, when the antihistamine terfenadine is coadministered with macrolide antibiotics which inhibit CYP3A4, the plasma levels of terfenadine increase and can cause toxic effects. This drug interaction can be rationalized on the basis that terfenadine is normally metabolized by liver and intestinal CYP3A4 to *tertiary*-butyl alcohol and then to a carboxylic acid metabolite. When formation of the final carboxylic acid metabolite is blocked by CYP3A4 inhibitors, the plasma levels of the parent drug (terfenadine) become sufficiently elevated to block cardiac potassium channels, which can lead to

arrhythmias. Thus, terfenadine was determined to be metabolized primarily by CYP3A4. Alternatively, P450 inducers can increase the rate of xenobiotic transformation. Induction lowers blood levels of a compound and can compromise therapeutic goals of drug therapy but in general does not cause an exaggerated response to xenobiotics.

Flavin-containing monooxygenases (FMOs) oxygenate the nucleophilic nitrogen, sulfur and phosphorous heteroatoms of xenobiotics (Klaassen 1996). FMOs are located in the endoplasmic reticulum of the liver, kidney and lung. In general, the reactions catalyzed by FMOs are mechanisms of detoxification. FMOs require NADPH and O_2 to carry out monooxygenation of a substrate (Figure 1.2). Humans and other mammals express five different FMOs (FMO1-5) in a species and tissue-specific manner. The various forms of FMOs are distinct gene products with different physical properties and substrate specificities. Many of the reactions carried out by FMOs can also be catalyzed by cytochrome P450s. In contrast to P450s, most FMOs are heat labile and can be inactivated above 50°C. This property can be used to distinguish the reactions catalyzed by FMOs from those catalyzed by P450s. The use of chemical inhibitors to ascertain the role of FMOs in oxidation of a substrate is often complicated by a lack of specificity of inhibitors to FMOs as a general class of enzymes.

Carboxylesterases are responsible for hydrolyzing a variety of xenobiotics (Klaassen 1996) (Figure 1.3). They are widely distributed throughout the body and are present in high levels in the endoplasmic reticulum, cytosol and plasma. The broad substrate specificity of these enzymes precludes the possibility of naming them for the reactions they catalyze. Thus, a systematic nomenclature remains to be established for these enzymes. Liver microsomes from all mammalian species contain at least one carboxylesterase but the exact number of carboxylesterases expressed in any one tissue or species is not known.

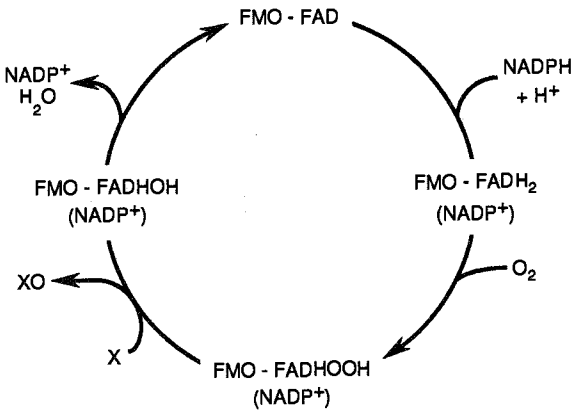


Figure 1.2 Catalytic cycle of flavin-containing monoxygenases. X and XO are the xenobiotic substrate and oxygenated product, respectively.

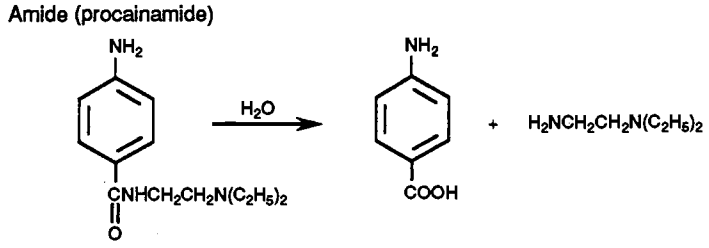


Figure 1.3 Example of a reaction catalyzed by carboxylesterases.

Phase II biotransformation involves reaction of a cofactor with a functional group present on a xenobiotic or added during reaction with Phase I enzymes (Klaassen 1996). Phase II reactions conjugate xenobiotics so that they are more polar and hence more excretable. Glucuronidation requires uridine diphosphate-glucuronic acid as a cofactor and is catalyzed by UDP-glucuronosyltransferases in the endoplasmic reticulum of the liver and other tissues. The UDP-glucuronosyltransferases are categorized into two gene families (UGT1 and UGT2) each capable on encoding multiple forms of UDP-glucuronosyltransferases. The C-terminus of all UDP-glucuronosyltransferases contains a membrane-spanning domain that anchors the enzyme into the endoplasmic reticulum. The enzyme faces the lumen of the endoplasmic

reticulum where it is ideally placed to conjugate lipophilic xenobiotics and their metabolites generated from P450 metabolism. The site of glucuronidation is usually an electron-rich nucleophilic heteroatom (O, N or S). Glucuronide conjugates of xenobiotics and endogenous compounds are polar and water-soluble. Hence, they are eliminated from the body in the urine or bile. However, glucuronidation can also represent an important event in the toxicity of xenobiotics, as seen in the development of bladder cancer from aromatic amines (Figure 1.4).

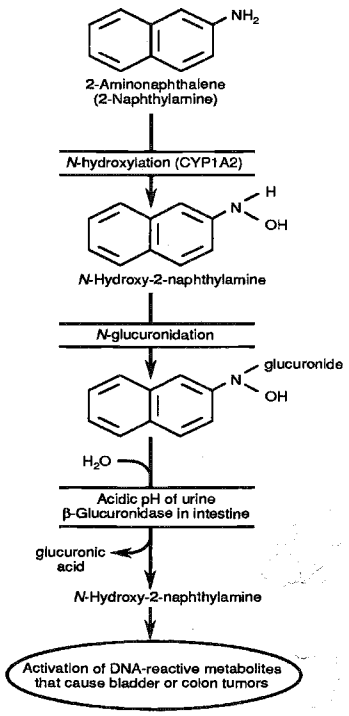
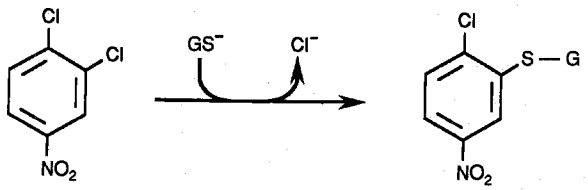


Figure 1.4 Example of the role of glucuronidation in the activation of xenobiotics.

Glutathione conjugation occurs with a wide variety of substrates through conjugation of a glutathione thiolate anion (GS^-) with the electrophilic atom of a xenobiotic (Figure 1.5). Glutathione conjugation is catalyzed by a family of glutathione-S-transferases (GSTs) which are present in high concentration in



1,2-Dichloro-4-nitrobenzene

Figure 1.5 An example of glutathione conjugation with an electrophilic carbon.

the endoplasmic reticulum and cytosol of most tissues. GSTs are dimers composed of identical or different subunits. Numerous subunits have been cloned and sequenced which forms the basis of the nomenclature for GSTs. Each enzyme is assigned a two-digit number to designate its subunit composition (e.g. 1-1 for a homodimer made up of two 1 subunits). The soluble GSTs are arranged into four classes designated A, M, P and T. The subunits in different classes share only about 30% identical in amino acid composition which prevents dimerization of subunits from different classes, while subunits in the same class are about 70% identical and can form heterodimers. The microsomal GSTs are distinct from the soluble enzymes. Two microsomal GSTs have been identified including one trimeric enzyme that conjugates xenobiotics with GSH and another that specifically conjugates leukotriene A_4 (a lipid epoxide derivative of arachidonic acid) with GSH to form leukotriene C_4 synthase.

Substrates for glutathione include an enormous array of electrophilic xenobiotics. Three common features of glutathione substrates include hydrophobicity, an electrophilic atom and the ability to react non-enzymatically with glutathione at some measurable rate. Glutathione conjugates formed in the liver can be excreted in the bile or they can be converted to mercapturic acids in the kidney and excreted in the urine. Thus, they represent an important detoxification route in the metabolism of xenobiotics. However, they can also enhance the toxicity of compounds by forming reactive toxic

metabolites or serving as transport vehicles to other organs where enzymes further metabolize the conjugate to toxic compounds.

Part I. Pyrrolizidine Alkaloids

Background

Pyrrolizidine alkaloids (PAs) are among the most prolific groups of plant toxins affecting livestock, wildlife and human health throughout the world (Seawright 1995, Huxtable 1989). More than 660 PAs have been isolated from over 6000 plant species (Xia et al. 2003). It is estimated that approximately 3% of the world's flowering plants contain toxic PAs (Logie et al. 1994, Smith and Culvenor 1981). PAs are largely hepatotoxins, capable of causing both acute and chronic toxicities (Mattocks 1986c). PAs can also affect extrahepatic organs such as the lungs and heart as well as initiate cancer development in a variety of animal models (Prakash et al. 1999, Mattocks 1986a, Mattocks 1986b).

Acute toxicity rarely occurs under natural conditions. It usually requires large doses of PAs, such as when PAs are rapidly absorbed by intraperitoneal or intravenous injection, and results in severe hemorrhagic necrosis of the liver and rapid death (Mattocks 1986c). The acute illness has been compared to the Budd-Chiari syndrome which presents with thrombosis of hepatic veins, leading to liver enlargement, portal hypertension and ascites. Clinical signs include nausea and acute upper gastric pain, acute abdominal distension, fever and changes in serum liver enzymes.

Chronic toxicity usually results in the development of veno-occlusive disease (VOD) of the liver which occludes small branches of the hepatic veins and causes ascites, edema, reduced urinary output and can lead to cirrhosis and death. VOD is highly characteristic of PA exposure and has been documented throughout the world (Huxtable 1989). However, because

chronic exposure to PAs usually leads to cirrhosis which can be caused by a number of conditions, chronic PA toxicosis may be clinically indistinguishable from other sources of hepatic injury (Mattocks 1986c).

After the liver, the lungs are the most common site of toxic action of PAs (Mattocks 1986c). Eleven-membered macrocyclic diesters such as monocrotaline are particularly active at producing toxicity in the lung. Clinical signs include alveolar edema and hemorrhage, proliferation of arterial walls and pulmonary hypertension. Chronic heart damage often occurs as a result of PA-induced lung damage.

In livestock, serum liver enzymes increase before development of recognizable lesions and are recommended to be measured if PA toxicosis is suspected (Craig et al. 1991, Pearson and Craig 1980). These include increased levels of glutamate dehydrogenase followed by increased amounts of alkaline phosphatase and γ-glutamyl transferase. Clinical signs of PA toxicosis include head pressing in horses and ammonia toxicity (Cheeke 1989). Elevated blood ammonia is due to impaired liver metabolism of amino acids and can cause spongy degeneration of the central nervous system, leading to displays of head pressing. Animals with chronic PA toxicity show marked ascites, due to a depression of serum albumin. Serum albumin plays an important role in maintaining osmotic relationships in the body. When albumin levels become unbalanced, this results in edema (low serum albumin) or dehydration (high serum albumin). Plasma and liver concentrations of vitamin A have also been found to be depressed in rats fed tansy ragwort (Moghaddam and Cheeke 1989). This could be due to an effect of PAs on the hepatic synthesis of retinol-binding protein or impaired biliary excretion. Vitamin A plays a key role in vision and animal growth so depression of this vitamin would alter those functions. In addition, elevated copper levels in the liver due to impairment of normal subcellular excretory mechanisms including defects in lysosomal elimination of cell waste (Cheeke 1991). Unfortunately,

animals have often consumed PA-containing plants for an extended period of time before signs of poisoning are clinically manifested (Mattocks 1986c).

The carcinogenicity of PAs and their metabolites has only been shown in animals, but it has been suggested that chronic, low-dose exposure to PAs has the potential to cause carcinogenic and/or teratogenic effects in people and livestock (Steenkamp et al. 2001). This may be a contributing factor to the high rates of liver cancer and cirrhosis seen in African and Asian countries. Toxic PA metabolites are persistent in aqueous media which enables them to access the nucleus and react with DNA, forming crosslinks both within DNA and between DNA and nucleoproteins (Prakash et al. 1999). It is thought that these metabolites disrupt the cell cycle by damaging key genes which control cell division leading to mitotic bypass and development of megalocytosis and/or cancer through continual synthesis of DNA and proteins (Prakash et al. 1999, Samuel 1975). A recent study found that low doses of retrorsine led to the development of multinucleated cells in a human hepatoma cell line in addition to failure in spindle formation and clumping of nuclear chromatin, suggesting the possibility of carcinogenic effects (Steenkamp et al. 2001).

Livestock Toxicity to Pyrrolizidine Alkaloids

The plants most frequently involved in livestock poisoning belong to the *Senecio* and *Crotalaria* genuses (Cheeke 1989). There are approximately 3000 plants species in the *Senecio* genus world-wide with over 100 of them containing hepatotoxic PAs (Stegelmeier et al. 1999). PAs from plants of the *Senecio* genus cause irreversible liver damage with clinical signs appearing as a consequence of impaired liver function (Cheeke 1988). The *Crotalaria* genus contains several hundred species of plants that produce PAs toxic to the liver and extrahepatic organs (Huxtable 1989). Most of these plants are concentrated in the tropical and subtropical regions of the world. Livestock

poisoning from *Crotalaria* species primarily leads to pulmonary damage with hepatic effects being less prominent.

Plants of the *Senecio* genus have been the cause of many toxicity syndromes (Cheeke and Shull 1985, Bull et al. 1968). *Senecio jacobaea* has been demonstrated to cause Winton disease in cattle and horses of New Zealand and Pictou disease in cattle and horses of Canada. Ingestion of *S. burchelli* and *S. latifolius* has been shown to cause Molteno horse disease or stomach staggers in horses and cattle of South Africa. *S. jacobaea* and *S. aquaticus* were found to cause enzootic cirrhosis of the liver in Holland. Great Britain and Ireland reported a series of livestock poisoning incidents in the early 1900s thought to be due to plants of the *Senecio* genus. In Germany, Schweinsberger disease of horses has been shown to be caused by *S. vernalis*, while zd'ar disease in horses of South Bohemia is caused by consumption of *S. erraticus*. In the 1930s, a disorder called walking disease of horses was identified in Nebraska, Colorado and Wyoming. One of the debated causes of this disease is consumption of *S. riddelii* and *S. longilobus* plants by infected animals. *S. douglasii* has also been reported to poison cattle in the southwest United States.

Extensive work has been done on the toxicity of *Senecio* spp. of PA-containing plants to livestock in the United States where their range of infestation is extensive (Figure 1.6) (Huan et al. 1998a, Craig 1995, Cheeke 1992, Craig et al. 1992b, Craig et al. 1992a, Craig et al. 1991, Cheeke 1989, Cheeke 1984). Tansy ragwort (*Senecio jacobaea*) was introduced to the United States in the late 1800s from the British Isles (Stegelmeier et al. 1999). It was first observed in Oregon in 1922 and has continued to spread throughout the northwest. It is an extremely abundant weed and is difficult to eradicate. It is not willingly eaten by livestock but may be eaten if pastures are in poor condition (as in times of drought), during early growth stages, or if it is incorporated into hay (Mattocks 1986c). Poisoning of cattle in the northwest

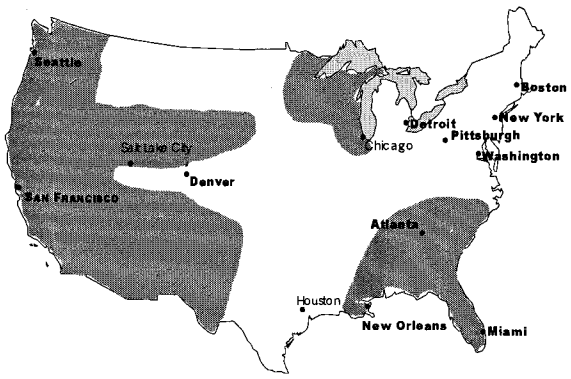


Figure 1.6 Areas of the United States affected by PA-containing plants.

by this plant results in major economic losses for livestock producers each year (Craig et al. 1992a).

Plants from the *Crotalaria* genus are also responsible for diseases in livestock (Bull et al. 1968). *Crotalariosis* (or stywesiekte) is a name given to a disease of cattle and horses in South Africa due to the effects of *C. burkeana* ingestion. In western Iowa and eastern Nebraska, 'Missouri bottom disease' of horses has been attributed to *C. sagittalis*. Jaagsiekte has been observed in sheep, horses and mules of South Africa and is thought to be due to the toxic effects of *C. dura*. *C. spectablis* has caused the loss of chickens, swine, cattle and horses in the southeast United States. Walkabout disease in horses was reported in Australia and is thought to be due to *C. retusa*.

In livestock, plants containing PAs are of major concern as they can lead to large economic losses for livestock producers (Stegelmeier et al. 1999, Cheeke and Huan 1995, Hooper 1978). Losses induced by plant poisonings can be direct or indirect. Direct losses are measured in annual mortality attributed to poisonous plants. Indirect losses include the cost of control measures (e.g. fencing and veterinary expenses), temporary or permanent non-utilization of toxic pastures and the diminished value of infested land (Kellerman et al. 1996). Toxicity problems occur across all continents, including the northwestern United States where tansy ragwort causes $20

million in livestock loss each year (Craig et al. 1991). In South Africa, the third most important cause of poisoning in livestock is the disease *seneciosis* which is caused by ingestion of *Senecio* plant species known to contain PAs (Kellerman et al. 1996). It accounts for 10% of cattle and 5% of small stock (such as sheep and goats) mortality due to plant poisonings. In a third world country with a struggling economy such as South Africa, these mortalities have a tremendous impact on farmers who depend on healthy livestock for food, clothing and as a means of transport (Christo Botha, personal communication).

There is a wide range in the susceptibility of animal species to PA-containing plants making extrapolation of data from animal studies of one species to another difficult. Individual susceptibility to toxicity is influenced by many factors including age, sex and species as well as other temporary factors such as biochemical, physiological and nutritional status (Stegelmeier et al. 1999). Especially at risk are adolescents and babies (Stegelmeier et al. 1999) since there is a higher susceptibility to toxicity in tissues which have a high concentration of rapidly dividing cells. For example, retrorsine was more toxic to 14 day old rats than to adults as observed by the lower liver weight and higher concentration of toxic metabolites in liver tissue of the 14 day old rats (Mattocks 1986c, Mattocks and White 1973). In addition, there are several reports of human infants developing VOD when they were treated with herbal teas or when their mothers used an herbal preparation during pregnancy (Huxtable 1989).

Some animal species show a distinct sex difference in susceptibility to PA toxicity, depending on the alkaloid studied (Mattocks 1986c). Human males are generally more susceptible than females based on their higher metabolic conversion of PAs in the liver or possibly because they are exposed to a larger amount of the contaminants as a result of higher food volume consumption. Rat liver microsomes exhibit a marked sex difference in *N*-oxidation of senecionine, owing to the presence of the male-specific CYP2C11 (Williams et

al. 1989a). CYP2C11 is more efficient at senecionine *N*-oxidation than CYP3A1 which appears to carry out both *N*-oxidation (detoxification) and formation of the pyrrole metabolite 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine or DHP (bioactivation) from senecionine. This suggests that female rats are more sensitive to PA toxicity than male rats due to their low rates of formation of *N*-oxide relative to DHP, which has been demonstrated previously (Mattocks 1986c).

Substantial variability exists amongst livestock species in response to the toxicity of PAs (Huan et al. 1998a, Winter et al. 1988). Pigs are quantitatively the most sensitive of the animals studied, followed by chickens, cattle, and horses. Sheep and goats are the most resistant. Cattle and horses develop chronic terminal hepatic disease after consuming as little as 5% of their body weight in tansy ragwort, while sheep are able to digest up to 300% of their body weight without adverse effects (Craig et al. 1991, Cheeke 1984). Mice require 150% of their body weight in plant material from *Senecio* plant species while rats and chickens require 50% and 5%, respectively (Hooper 1978). Feeding trials by Cheeke et al. have yielded similar results for these animals and have also measured the lethal dose for several other rodents and poultry (Cheeke 1984, Cheeke and Pierson-Goeger 1983). Gerbils were the most resistant, consuming 36 times their body weight in PA-containing plant material. Hamsters had similar body weight ratios as sheep and goats, while guinea pigs and rabbits were slightly more sensitive to toxicity. Goeger et al. (1982) demonstrated that goats are more resistant than cattle to PA poisoning, requiring over 100% of their initial body weight in PA-containing plant material. Young chickens and turkeys were both extremely sensitive to PA poisoning (Cheeke and Pierson-Goeger 1983).

Several factors may explain species differences in susceptibility to PA toxicity. Bioactivation and detoxification processes in the liver including the rate of formation of toxic and non-toxic products, the rate of PA hydrolysis, and the rate of glutathione conjugation to toxic metabolites and excretion, can be

important in determining the ultimate toxic response in the animal (Williams et al. 1989a). In addition, resistant species could abate PA toxicity by absorbing more PA into the enterocytes of the small intestine for subsequent excretion in the feces or by metabolizing PAs to non-toxic moieties in the rumen.

It is thought that a major factor in species susceptibility to PAs is the ability of an animal to metabolize the parent compound into the reactive, electrophilic pyrrole metabolites (Cheeke 1994). Resistant species, such as sheep are said to have lower rates of pyrrole formation than susceptible species such as cattle, suggesting that PAs are bioactivated at a slower rate in resistant species. However, an *in vitro* comparative metabolism study has found that there is not a strong correlation between the production of DHP and susceptibility of an animal to PA toxicity (Huan et al. 1998a). This study also discovered that resistant animals such as sheep and hamsters converted the highest proportion of parent compounds to their DHP or *N*-oxide products, whereas susceptible species such as cattle and rats had a low metabolic capability toward the parent compound. Another *in vitro* study found that resistance of the guinea pig to PA poisoning may be due to its resistance to the toxic effects of DHP rather than to low DHP formation (Winter et al. 1988).

Another possible mechanism for the differential sensitivities of various species to PA intoxication could relate to differences in the relative amounts of PAs that reach the livers of each species as a result of intestinal metabolism of PAs. Biotransformational enzymes present in the gastrointestinal tract can play an important role in metabolism of xenobiotics and drugs (Cheeke 1994, Smith 1992). Cytochrome P450-mediated biotransformation can occur in intestinal epithelial cells (enterocytes), reducing the amount of parent drug or chemical available for absorption and thus limiting their beneficial or toxicological effects (Hall et al. 1999, Zhang et al. 1999, Kaminsky and Fasco 1991). In some cases, the reactive metabolites covalently bind to enterocyte macromolecules (Kolars et al. 1994). Since the enterocytes normally slough off of the villus tips after a few days, the adducts are harmlessly excreted in

the feces, reducing the toxicity of the parent chemical. A similar mechanism could occur in the intestine in the case of PAs, hence limiting an individual's exposure to toxic PAs through intestinal first-pass metabolism. There is suggestive evidence, for example, that reactive metabolites of the PA heliotrine can react with and inhibit enzymes present in the intestinal mucosa (Karimov et al. 1995).

Differences in ruminal metabolism of PAs observed in sheep (resistant) and cattle (susceptible) rumen fluid is another major factor in susceptibility differences to PA toxicity. Early work on the role of ruminal microbes in PA metabolism involved the development of a new surgical technique for chronic portal vein infusion (Blythe and Craig 1986, Craig et al. 1986) and the isolation of large amounts of PAs by extraction from plant material (Craig et al. 1984). With these developments, actions in the sheep liver were isolated from actions in the rumen in a series of acute (Craig et al. 1985) and chronic (Craig et al. 1987) PA infusion experiments. Thus, the direct effect of PAs on the liver of resistant sheep and susceptible cattle was evaluated. Both species developed similar clinical (anorexia, adypsia, depression and abdominal distension and straining), enzymological (γ-glutamyl transpeptidase, alkaline phosphatase and glutamic dehydrogenase), and histological (post-sacrifice) evidence of liver toxicosis (Craig et al. 1987, Craig et al. 1985). The results suggested an important role of the digestive system in the protection of sheep from PA toxicosis. Thus, attention was focused on the possibility that ruminal microbes in sheep can degrade the alkaloids before they become systemic poisons.

A series of *in vitro* experiments were then conducted comparing the degradation capabilities and rates of sheep vs. cattle rumen fluid utilizing an artificial rumen technique with serum bottles (Craig et al. 1992a, Wachenheim et al. 1992b). In these experiments, it was found that sheep rumen fluid rapidly degraded PA within 2-6 hours, while bovine rumen fluid required 24-48 hours. This suggests that sheep rumen fluid is able to degrade PAs approximately ten times more efficiently than cattle rumen fluid. Sheep rumen

fluid was found to contain 3.0 x 10^7 PA-degrading bacteria per ml, whereas bovine rumen fluid contained only 1.1 x 10^7 PA-degrading bacteria per ml when counted through most probable number analysis (Wachenheim et al. 1992b). The lower number of microorganisms in the bovine species is believed to be one of the main factors in their susceptibility to the toxin, in that there is not rapid detoxification prior to ruminal absorption of the toxin. Pasteurization of the sample prior to incubation eliminated the detoxification phenomenon, suggesting that microorganisms were essential to the process.

Further studies have confirmed that a small group of bacteria were responsible for the PA detoxification (Craig 1995, Craig et al. 1992b). A series of enrichments of whole rumen fluid from sheep that were consuming tansy ragwort resulted in the isolation of a mixed culture (L4M2) that degraded a mixture of PAs isolated from tansy ragwort in less than 12 hours. Currently, the L4M2 culture exists as six bacterial species that act as a consortium to degrade PAs. Recent work has demonstrated that L4M2 metabolizes a variety of structurally different PAs including jacobine, senecionine and seneciphylline which are 12-membered macrocyclic PAs; monocrotaline which is an 11-membered macrocyclic PA; heliotrine which is a monoester; and lasiocarpine which is an acyclic diester (Hovermale and Craig 2002). Heliotrine and lasiocarpine have a stereochemistry at C-7 which is opposite to that of all the macrocyclic PAs tested. The heliotridine base structure is a stereoisomer of the retronecine base structure of the other molecules. Thus, L4M2 is capable of metabolizing structurally different PAs, both in terms of stereochemistry and overall structure (macrocyclic versus acyclic). This indicates that L4M2 would offer protection from a wide variety of PA containing plants, in addition to tansy ragwort. The ability of L4M2 to metabolize so many PAs, which are common in poisonous plants throughout the world, makes it economically beneficial. Rather than being of limited usefulness only in the Pacific Northwest, a probiotic containing L4M2 could be marketed as prevention against several toxic plants throughout the world. In summary, this

information suggests that species susceptibility is a complex interaction involving species specific differences in both pre- and post-absorption metabolism (Stegelmeier et al. 1999).

Human Toxicity to Pyrrolizidine Alkaloids

Pyrrolizidine alkaloid poisoning in humans is a significant toxicity problem in many areas of the world. Pyrrolizidine alkaloids may enter the human food supply by ingestion of contaminated grain, by consumption of animal products such as meat, milk, eggs and honey, or as contaminants in herbal remedies or the active ingredient used in traditional medicine (Cheeke 1991). The number of cases of pyrrolizidine poisoning in humans recorded each year is likely to be inaccurate due to underreporting, misdiagnosis by medical personnel not trained to recognize the toxicity symptoms which are similar to those of other diseases, and a lack of medical inquiry stemming from the misconception that natural compounds are healthful rather than potentially harmful (Huxtable 1989).

Exposure to PA-containing plants and contaminated grains can present a serious health hazard to developing countries. Outbreaks typically occur when large numbers of people ingest contaminated food grains and develop VOD of the liver. Incidences of human poisoning have been recorded in the scientific literature since 1918 (Willmot and Robertson 1920), with the most exceptional cases occurring in Afghanistan and India. A total of 7,800 out of 35,000 reported cases of people eating contaminated food grains developed VOD (Tandon et al. 1978, Mohabbat et al. 1976) in Afghanistan, while 28 people out of 67 reported cases of VOD died in a poisoning incident in India (Tandon et al. 1976).

Periodic exposure to small amounts of PAs in animal products is likely to occur, especially in countries where pyrrolizidine toxicity is a problem in livestock. The most frequently encountered source of PAs found amongst

animal products is milk of animals that have ingested PA-producing plants. Liver damage has been found in rats fed milk (7.5 ng PA/g dry weight) from lactating goats on a diet containing tansy ragwort (Goeger et al. 1982). Transference of PAs into human breast milk has been suspected as the cause for VOD in infants for whom no history of herbal administration could be elicited (Huxtable 1989). A recent report has indicated that chickens can transfer PAs to their eggs by eating contaminated grain (Edgar and Smith 2000). Analysis of the feed confirmed the presence of PAs which were found in the eggs at a concentration of 1.2 to 9.7 µg total PA/egg. Residues of PAs have not been found in meat from animals ingesting alkaloid-containing plants, since the compounds are metabolized into reactive pyrrolic intermediates with relatively short half-lives and are cleared rapidly from the tissues. Slaughter would have to occur within a few hours of grazing on contaminated pasture to be toxic to humans (Huxtable 1989). Honey has been found to contain PAs at levels of 0.3-3.9 mg/kg (Deinzer et al. 1977), making those who consume large amounts of honey at risk for toxicity. Although these exposure incidences in and of themselves may not cause immediate toxic effects in humans, the cumulative and irreversible nature of pyrrolizidine alkaloid toxicity highlights this as an important source of added exposure to PAs.

While remaining a staple of traditional medicine in developing countries to treat a wide range of diseases and conditions, the use of PA-containing herbal remedies, teas and dietary supplements has increased in industrialized countries in recent years with a concomitant rise in the number of pyrrolizidine poisonings in those nations (Prakash et al. 1999). PA poisonings in the United States include a woman who developed VOD after consuming medicinal tea from Ecuador over a period of six months (Huxtable 1989). Mexican Americans also make an herbal cough medicine from a plant called gordolobo yerba that looks similar to the PA-containing plant *Senecio longilobus*. Children who consume medicine tainted with *S. longilobus* have been admitted to the hospital and died from VOD.

Plants of the genus *Symphytum* are of major concern in industrialized nations due to the widespread use of *S. officinale* (common comfrey) as an herbal supplement (Huxtable 1989). The claims made for using comfrey are as a treatment for inflammatory disorders, as a digestive aid and as a general panacea (Stickel and Seitz 2000). As people may take up to six capsules per day for many months, comfrey represents a major source of exposure to pyrrolizidine alkaloids (Huxtable 1989). The U.S. Food and Drug Administration issued a letter in July of 2001 to dietary supplement manufacturers, stating that "while information is generally lacking to establish a cause-effect relationship between comfrey ingestion and adverse effects in humans...the agency strongly recommends that firms marketing a product containing comfrey or another source of PAs remove the product from the market...The agency advises that it is prepared to use its authority and resources to remove products from the market that appear to violate the [Federal Food, Drug and Cosmetic] Act." The German government has recently set a limit for daily exposure to pyrrolizidines and *N*-oxides at 0.1 µg pyrrolizidine per day for not more than six weeks in one year (Stegelmeier et al. 1999). Similar regulations have been proposed in other countries such as Britain and Australia.

Hepatic Metabolism of Pyrrolizidine Alkaloids

Metabolism of PAs occurs by three main pathways in the liver (Mattocks 1986c). First, PAs can be oxidized to *N*-oxides by the Phase I cytochrome P450 and/or flavin-containing monooxygenase (FMO) enzymes (Figure 1.7). Second, PAs can be hydrolyzed to necine base and necic acid moieties via carboxylesterases. Third, the PA can be oxidized by cytochrome P450 (P450) monooxygenases to reactive carbonium ion intermediates which are unstable and either undergo rapid hydrolysis to necine pyrroles and/or covalently bind to DNA and other cellular nucleophiles. Pyrrole esters have been shown to

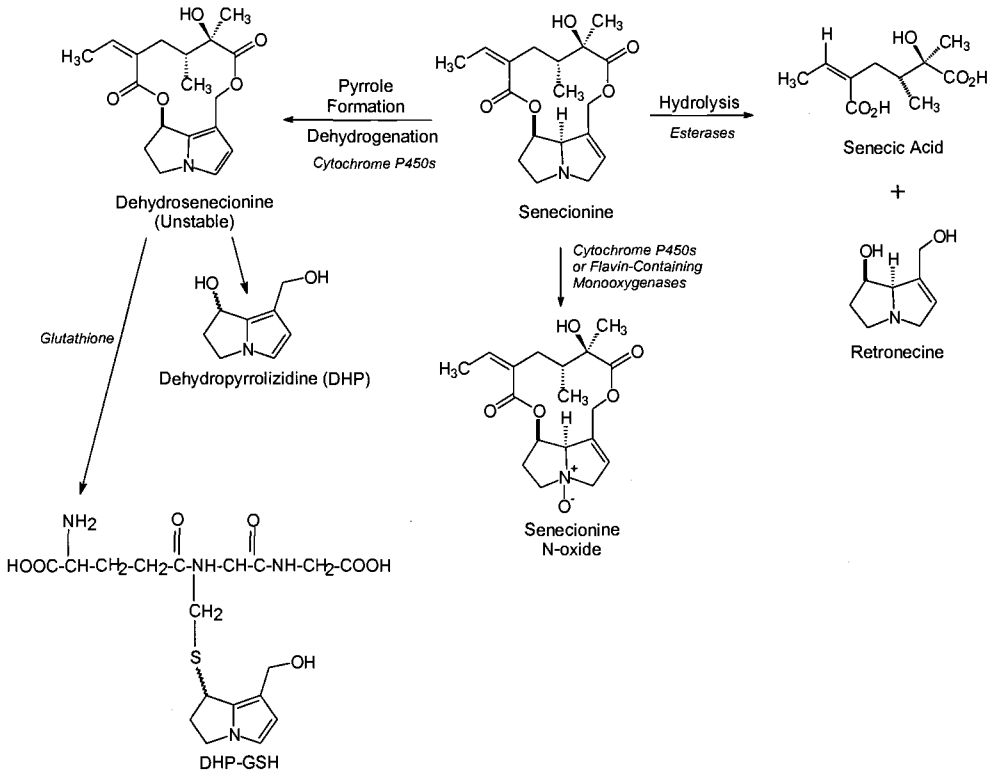


Figure 1.7 Major pathways of hepatic PA metabolism.

react with glutathione (GSH) to form a DHP-GSH conjugate which increases the water solublility of the pyrrole. The conjugate is subsequently excreted into the bile or sinusoidal blood and ultimately into the urine (Yan and Huxtable 1995). Alternatively, the soluble DHP-GSH conjugate can serve as a transport vehicle to other organs such as such as the lung, in which toxicity can be elicited if the pyrrole is released from GSH (Cheeke and Huan 1995). Identification of the enzymes involved in the metabolism of PAs and their role in *N*-oxide, necic acid plus necine base or DHP production is an important step in understanding the potential hazards of PAs in animals and humans (Miranda et al. 1991a).

PAs are metabolically converted to *N*-oxides through hepatic cytochrome P450s or flavin-containing monooxygenases, depending on the species

involved. PA *N*-oxides are relatively unreactive and are believed to be excreted unchanged in the urine due to their water solubility. Evidence based on optimal pH, thermal stability, and enzyme inhibition data suggested that senecionine *N*-oxidation was carried out largely by FMOs in the liver, lungs and kidney of guinea pigs, a resistant species (Miranda et al. 1991b). In guinea pig liver, senecionine *N*-oxide formation far exceeded the rate of DHP formation, which was catalyzed by cytochrome P450s. These findings are of great significance in explaining the resistance of guinea pigs to PA intoxication. FMO was also responsible for the *N*-oxygenation of senecionine in purified pig liver microsomes and partly so for rat liver microsomes (Williams et al. 1989b). In addition, chemical inhibition data suggest that conversion of senecionine to its *N*-oxide in sheep and hamster liver microsomes is catalyzed mainly by FMOs (Huan et al. 1998a). As discussed below, CYP3A4 catalyzes the *N*-oxidation of senecionine in human liver microsomes (Miranda et al. 1991a). In plants, CYP1A1 has been shown to be involved in the formation of *N*-oxides from retrorsine in avocado microsomes (Couet et al. 1996).

Studies have challenged the notion that *N*-oxidation of PAs is an irreversible detoxification process. One study incubated retrorsine *N*-oxide with human and rat liver microsomes and found that it was partially converted back to the parent alkaloid or to DHP (Couet et al. 1996). This suggested that *in vivo* formation of *N*-oxides may be an indirect source of toxicological challenge. The mode of PA introduction can have a dramatic influence on toxicity (Mattocks and White 1971). When rats were given retrorsine *N*-oxide intraperitoneally, pyrroles accumulated in the liver at a slower rate than when given orally (Mattocks 1972). In addition, evidence exists that PA *N*-oxides are reduced to their bases in sheep rumen fluid (Lanigan and Smith 1970) or the gut (Mattocks and White 1971). This suggests that reduction of the *N*-oxide to its base is a key step in metabolic activation of *N*-oxides. These data are important in terms of ingestion of pyrrolizidine-containing plants because the *N*-oxide form of a PA often exist in greater quantities in the plant than the

parent alkaloid (Mattocks 1986c). Moreover, the European cinnabar moth (*Tyria jacobaeae*) was found to convert necine bases to their corresponding *N*-oxides in the hemolymph with an enzyme called senecionine *N*-oxygenase (Naumann et al. 2002). This insect has been used as a biological control agent in tansy-infested fields as they feed solely upon the PA-containing plant *Senecio jacobaea*. Senecioine *N*-oxygenase allows the larvae to accumulate *N*-oxides which are then used as predation deterrents. This provides further evidence that *N*-oxides may not be completely inert compounds.

Carboxylesterases catalyze the hydrolysis of PAs to necine base and necic acid moieties which are generally described as detoxification products. Steric factors prominently influence the susceptibility of PAs to hydrolysis, with short and unbranched acid chain esters being more easily hydrolyzed than those that are sterically hindered (Mattocks 1982). In fact, the ease of hydrolysis of the parent alkaloid has been shown to be inversely proportional to the levels of pyrrolic and *N*-oxide metabolites produced for a given alkaloid (Mattocks and Bird 1983).

Sheep have shown a large influence of esterases on senecionine metabolism, while hamsters have shown less of an influence (Huan et al. 1998a). Esterase hydrolysis also accounted for the majority of monocrotaline metabolism in the guinea pig, while the rat displayed no esterase activity (Dueker et al. 1992a, Dueker et al. 1992b). Therefore, metabolism by esterases may contribute to the resistance to PA toxicity seen in sheep and guinea pigs since a high rate of esterase hydrolysis in PA metabolism would decrease the proportion of PAs that are available for bioactivation to toxic metabolites.

However, the PA jacobine is unusually toxic to guinea pigs. Intrigued by this observation, two forms of hepatic carboxylesterase were purified from guinea pigs to investigate the role of esterolytic cleavage of PAs in the metabolism of jacobine (Chung and Buhler 1995). One form of purified carboxylesterase hydrolyzed jacobine and senecionine at rates of 4.5 and

11.5 nmole/min/mg protein, respectively. This implied that jacobine was less prone to esterolytic cleavage than senecionine and that more quantities of jacobine are available for bioactivation to DHP. Guinea pig liver microsomes converted jacobine to DHP and jacobine *N*-oxide at rates of 0.33 and 0.10 nmole/min/mg protein (DHP/*N*-oxide ratio of 3.16). Senecionine was converted to DHP and senecionine *N*-oxide in liver microsomes at rates of 0.46 and 0.87 nmole/min/mg protein (DHP/*N*-oxide ratio of 0.53). Therefore, it was concluded that the combination of high pyrrole and low *N*-oxide formation, together with relatively little carboxylesterase-catalyzed hydrolysis are the major factors responsible for the toxicity of jacobine in guinea pigs.

Liver cytochrome P450s oxidize pyrrolizidine alkaloids to reactive necine pyrroles. Enzymatic oxidation of PAs by P450s is centered at C-8 and results in the production of a bifunctional intermediate with reactive electrophilic centers at C-7 and C-9 by conjugation of the pyrrole's nitrogen lone pair (Figure 1.8). The toxicity of PAs is thought to be due to the covalent binding of necine pyrroles to DNA or other cellular nucleophiles. Covalent binding leads to disruption of the cell cycle, development of megalocytosis and eventually cancer. The covalent binding of pyrroles to some cellular components is

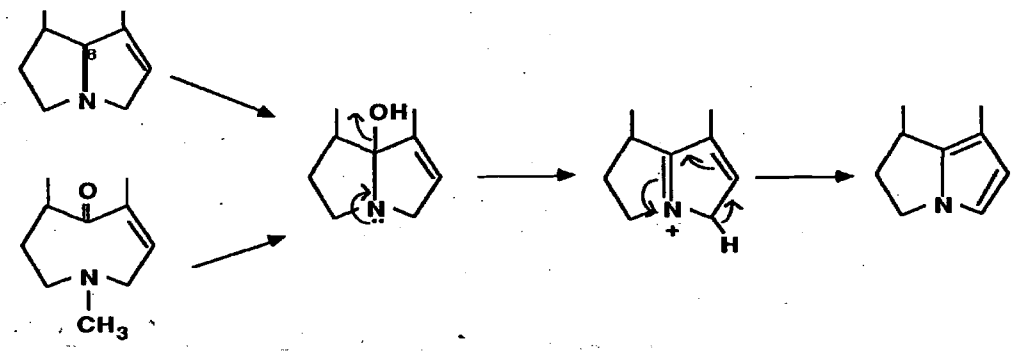


Figure 1.8 Mechanism for production of the pyrrole intermediate. It involves an initial CYP-mediated hydroxylation of the PA at C8 producing a chemically unstable carbinolamine which spontaneously loses the hydroxyl group, then a proton producing dehydropyrrolizidine as the final product.

potentially labile so that these tissue-bound conjugates can also serve as a reservoir of toxic PA metabolites which may therefore promote latent, progressive toxicity after elimination of the initial dose of PAs (Seawright 1995, Mattocks 1986c).

The *in vitro* metabolism of PAs by human liver microsomes has been studied by Miranda et al. (1991b). The enzyme activity of liver microsomes prepared from seven individual human donors towards senecionine was determined. In all individuals, DHP production was higher than that of *N*-oxide. A strong correlation ($r^2 = 0.99$) was obtained when DHP formation was plotted versus *N*-oxide formation, suggesting that both metabolites were formed from the same enzyme or enzymes under similar regulatory control. Based on rat studies (Williams et al. 1989a) it was suspected that CYP3A4, the human ortholog of rat CYP3A1, played a major role in PA bioactivation in human liver.

To confirm this hypothesis, specific inhibitors of CYP3A4 (gestodene, troleandomycin (TAO) and anti-human CYP3A4 IgG) were added to microsomal incubation mixtures. Gestodene, TAO and anti-human CYP3A4 markedly reduced the rate of formation of DHP and *N*-oxide. The rate of DHP and *N*-oxide formation correlated well with the rate of oxidation of nifedipine (a specific CYP3A4 substrate) and relative content of CYP3A4 as determined by western blotting. These correlations confirm the hypothesis that in humans the same enzyme, CYP3A4, catalyzes both DHP and *N*-oxide formation from senecionine. CYP3A is the most prevalent P450 isoform in the liver of humans (up to 60% of total P450) and other mammalian species and is inducible by many compounds (Guengerich 1989). This is an important consideration in the degree of PA toxicity elicited at a given point in time, in that a temporary induction of the P450 isoenzymes involved in PA metabolism from other compounds could result in more pyrrole formation if concurrent PA ingestion occurs.

Cytochrome P450s have also been shown to be the enzyme(s) responsible for PA bioactivation in other species. One study investigated the role of CYP3A and CYP2B in senecionine metabolism in sheep and hamster liver microsomes (Huan et al. 1998a, Huan et al. 1998b). Immunoinhibition by anti-sheep CYP3A and anti-sheep CYP2B showed that CYP3A and not CYP2B isoforms were responsible for most of the DHP formation in sheep and hamsters. The chemical inhibitors gestodene and TAO further confirmed that CYP3A was primarily responsible for senecionine bioactivation to DHP. Williams et al. (1989a) found that senecionine was primarily metabolized to DHP by CYP3A2 in rats, while CYP2C11 carried out the majority of senecionine *N*-oxidation. It was also found that CYP3A was responsible for the bioactivation of monocrotaline in rat liver microsomes (Reid et al. 1998b). CYP1A1/2, 2B1/2 and 2E1 proteins from liver microsomes were increased in response to retrorsine exposure in rats, suggesting that one or more of these enzymes may be involved in retrorsine metabolism (Gordon et al. 2000). Orthologs of CYP2B1 and CYP3A have also been suggested to play important roles in the bioactivation of senecionine to DHP in guinea pigs (Miranda et al. 1992). Further research in guinea pigs showed that CYP2B appeared to play a more significant role in senecionine bioactivation than CYP2C or CYP3A (Chung et al. 1995).

A range of toxicity exists for individual PAs as a result of oxidative metabolism by P450s. The degree of toxicity of an alkaloid correlates with the complexity of PA esterification, with cyclic diesters being the most hepatotoxic, followed by acyclic diesters which are more toxic than monoesters (Figure 1.9) (Steenkamp et al. 2001, Cheeke and Huan 1995). The two ester side groups on macrocyclic diesters are not capable of moving and blocking the reaction center at C-8, explaining their relatively high susceptibility to microsomal dehydrogenation and production of pyrroles. Alternatively, non-cyclic diesters, which are capable of exerting much hindrance at C-8 through movement of

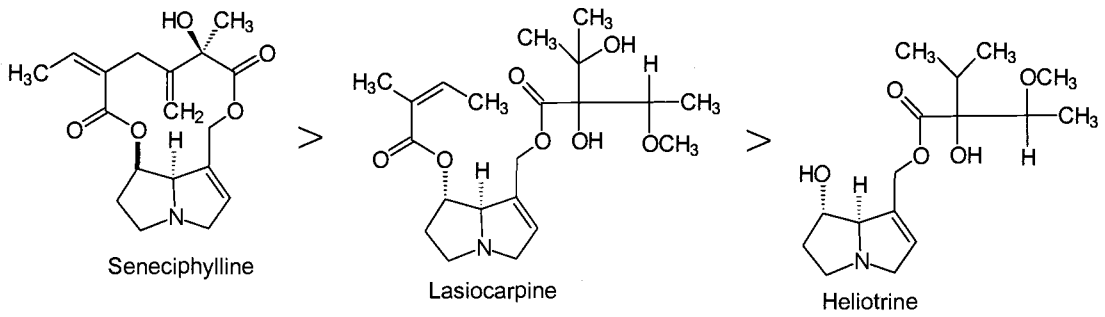


Figure 1.9 The toxicity of pyrrolizidine alkaloids is based on the structure attached to the C7 and C9 positions. Macrocyclic diesters > acyclic diesters > monoesters.

their ester side groups, have low pyrrole production (Winter et al. 1988, Mattocks 1986c, Mattocks and Bird 1983), and therefore generally elicit a less toxic response. Other structural features required for toxicity include a 1,2 double bond in the necine base and a branched ester group (Mattocks 1986c).

Glutathione conjugation is an important route of detoxification of PAs. Conjugation of PAs with GSH occurs at the C-7 hydroxyl of pyrroles. One study found that the reaction between GSH and reactive dehydropyrroles occured rapidly in the absence of glutathione-S-transferase (GST) enzyme (Reed et al. 1992). Thus, the nonenzymatic conjugation of PAs with glutathione plays a significant role in the detoxification of PAs. It has not been determined definitively whether or not the conjugation of PA metabolites is also catalyzed by GST isoforms. Since the GSH-pyrrole and cysteine-pyrrole conjugates of the PAs are considered to be nontoxic (Reid et al. 1998a), such conjugation reactions would diminish PA concentrations available for liver bioactivation. One study found that GSH depletion resulted in an increase in the release of active pyrroles from metabolism of monocrotaline in isolated, perfused rat liver (Yan and Huxtable 1995). Further, DHP formation was significantly depressed in sheep and hamster liver microsome incubations containing 2.0 mM GSH (Huan et al. 1998a). This suggests that when adequate GSH is present, GSH is capable of directly reacting with pyrrolic metabolites thereby decreasing their potential for toxicity.

Part II. Ergot Alkaloids

Background

The culture of grasses, their importance as livestock forage, and their potential for toxicity to humans and livestock were recognized early in human civilization (Bacon 1995). Toxicity caused by endophyte-infected grasses has been recorded since biblical times but it wasn't until 1977 that a fungal endophyte was associated as the cause of toxicity exhibited in livestock consuming endophyte-infected tall fescue (Bacon 1995, Bacon et al. 1977). Tall fescue (*Festuca arundinacea*) probably entered the United States as a contaminant in grass seed imported from Europe. It has become the most widely grown pasture grass in the southeast and northwest USA, occupying 35 million acres (Fribourg et al. 1991). It is currently extending its range worldwide to Europe, Asia and South America.

Tall fescue is preferred over other grasses due to characteristics produced from infection with the endophytic fungi *Neotyphodium coenophialum* including its ease of establishment, wide range of adaptation, tolerance of abuse, pest and drought resistance, good seed production and excellent appearance when used as turf grass (Hoveland 1993). In turf grass production, where animal performance is not a factor, endophyte infection is desirable as it results in improved growth (particularly when competing with other grass species), an increase in phenotypic variation (Hill et al. 1990), and resistance to environmental stresses (Hoveland 1993, Funk et al. 1985). The endophyte co-evolved with its grass host and is non-parasitic. Thus, a mutualistic relationship is maintained (Bacon and Siegel 1988).

A drawback of endophyte-infected tall fescue grasses is the production of ergot alkaloids and other toxins that adversely affect livestock that consume

infected plant tissue (Figure 1.10). Synthesis of these compounds is derived from oxidative reactions between tryptophan and dimethylallylpyrophosphate to form lysergic acid by the fungus (Garner et al. 1993). The ergopeptides are then produced via amide formation with an amino group of an amino acid to make up the cyclol portion of the molecule. Ergovaline has been identified as the most abundant ergot alkaloid produced by the fungus (Yates and Powell 1988), making up 84-97% of the total ergopeptide content (Lyons et al. 1986). Therefore, this alkaloid is targeted for diagnostic testing of grass samples to determine if they are safe for livestock consumption. However, lack of affordable, large quantities of this alkaloid presents a roadblock in definitive toxicity studies. Therefore, other ergot alkaloids with a similar chemistry are often used in place of ergovaline for research (Garner et al. 1993).

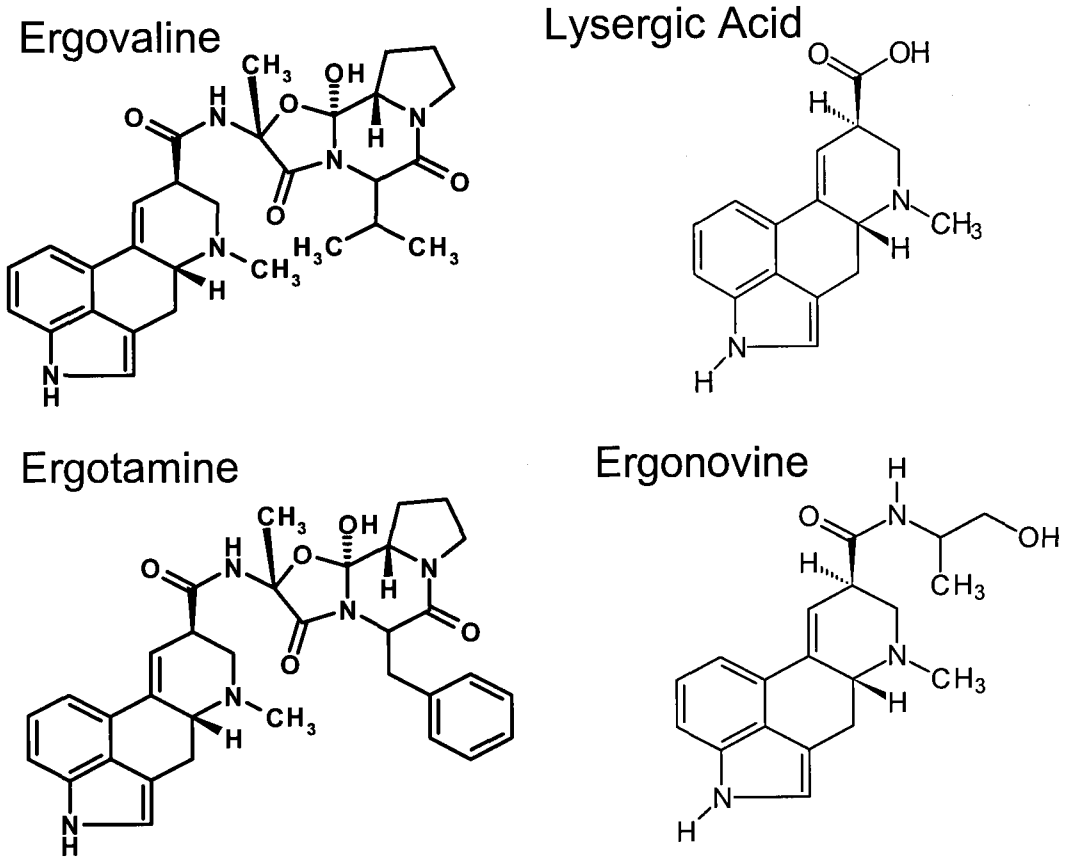
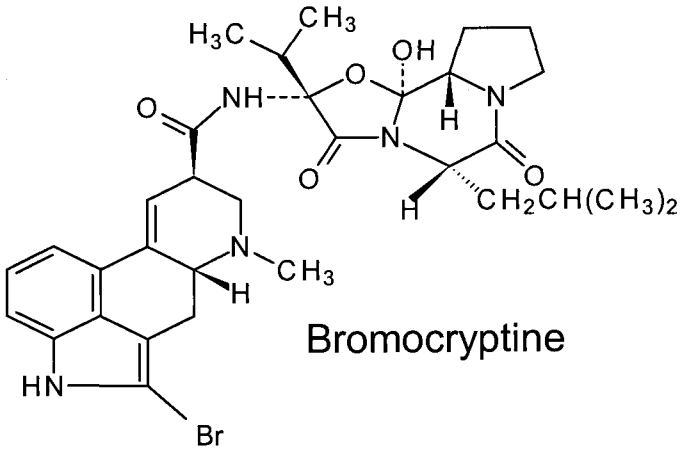
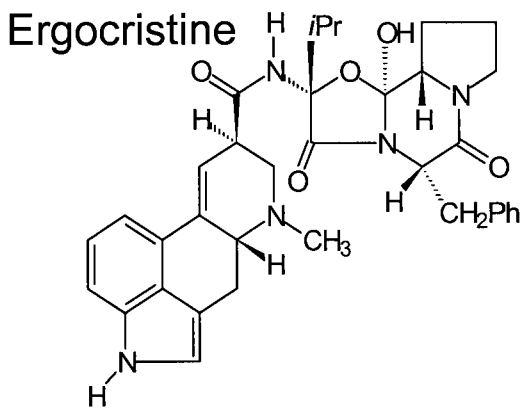
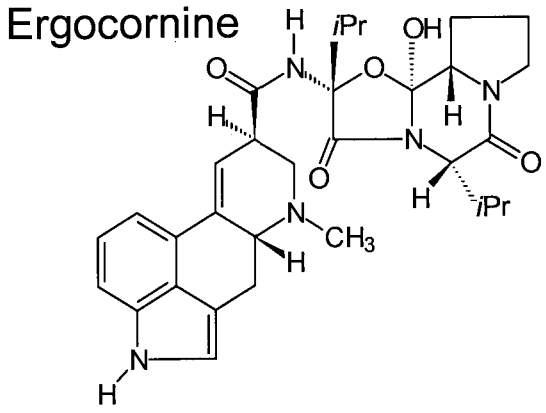
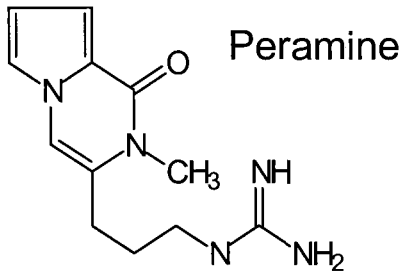


Figure 1.10 Chemical structures of ergot alkaloids.









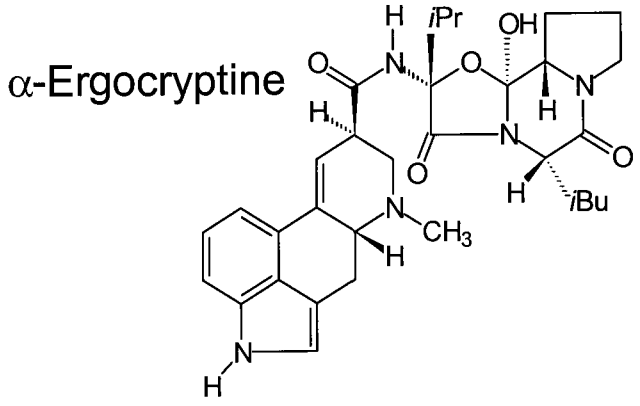


Figure 1.10 (Continued) Chemical structures of ergot alkaloids.

Endophyte-infected plants are toxic to insects and animals that ingest plant material containing alkaloids produced by the fungus. The pest resistance exhibited by endophyte-infected grasses is due to the effects of peramine and lolitrem toxins on the digestive system and distribution of herbivorous insects (Latch 1993). Lolitrem toxins present in perennial ryegrass (*Lolium perenne*) infected with the fungus *Acremonium lolii* modify the permeability of cations in the membrane lining the gut epithelium. This causes an increase in osmotic pressure which loosens the epithelium of connective tissues and results in complete digestive failure. In addition, it has been found that insects lay fewer eggs on endophyte-infected (E+) plants than on endophyte-free (E-) plants probably due to the fact that insects target E- over E+ plants for consumption (Latch 1993, Rowan et al. 1990). Nematodes are also influenced by the roots of endophyte-infected plants, as fewer nematodes were found in the soil near roots of E+ than E- tall fescue plants (Latch 1993). This lead to the conclusion that the alkaloid deterrents are also exuded from the roots.

The three disorders associated with consumption of endophyte-infected tall fescue in livestock are summer syndrome, fescue foot and fat necrosis. Clinical signs of 'summer syndrome' or 'summer slump' include poor weight gain, intolerance to heat, excessive salvation, rough hair coat, elevated body temperature, nervousness, low milk production and reduced conception rates (Hoveland 1993, Hoveland et al. 1983). Increased rectal temperature, respiratory rate and heart rate have been observed in cattle grazing E+ fescue (Hemken et al. 1981, Hemken et al. 1979). The latter two may be adaptive mechanisms to dissipate energy, particularly in areas like the southeast where summer temperatures intensify these changes (Thompson and Stuedemann 1993). This disorder is the largest cause of economic loss to livestock producers both because of its wide range of physiological effects on cattle and because cattle from the southern region of the US often bring reduced prices at the market place due to the predominance of E+ fescue in that region (Schmidt and Osborn 1993). Fescue foot is associated with colder

temperatures and begins as a red line at the coronary band of the hoof followed by gangrene, necrosis and eventually sloughing of the affected hoof (Thompson and Stuedemann 1993). Other affected extremities include the tips of the ears and the end of the tail.

Ergot alkaloids act as α-adrenergic agonists, which stimulates smooth muscle cell contraction and results in vasoconstriction (Thompson and Stuedemann 1993). When animals are subjected to extreme temperatures and consume E+ feed, they have trouble regulating blood flow to their extremities which can result in tissue ischemia, necrosis and sloughing of these body sections in the case of cold weather, and an inability to properly cool these body sections in the case of hot weather. Vasoconstriction can even result in strokes in some extreme cases. Lysergamide (or lysergic acid amide) has been shown to have vasoconstrictive properties in bovine vasculature, at doses within range of those expected when animals consume E+ fescue (Oliver et al. 1993). Similar responses have been found with ergonovine and ergotamine in cattle (Oliver et al. 1992, Oliver et al. 1990). In addition, the levels of the thermoregulatory hormone triiodothyronine (T_3) were found to increase in steers and in cows nursing calves receiving i.v. bolus doses of ergonovine maleate and ergotamine tartrate (Browning et al. 1998). Thyrotropin (T_4) levels were also increased in the cows. Thyroid hormones are responsible for increasing basal metabolic rate with concomitant heat production (Cunningham 1997). Thus, an increase in T_3 and T_4 would lead to heat intolerance from animals on E+ fescue containing these alkaloids.

Ergot alkaloids also act as dopamine agonists which inhibits the production of prolactin and results in diminished lactation (Oliver 1997, Larson et al. 1994). Prolactin suppression results in reduced milk yield in prepartum cattle but has no effect once lactogenesis occurs (Thompson and Stuedemann 1993). However, lactational yield is a complex balance between hormone levels and feed intake. One study showed that Holstein cows on toxic ryegrass-fescue hybrids had decreased milk yields compared to cows on non-

toxic grass, most likely due to reduced feed intake for cows on the toxic grass (Hemken et al. 1979). In addition, calf birth weight of beef cows on E+ fescue was not affected but milk production was reduced 100 days postpartum by 50% (Schmidt et al. 1986). The rough hair coat seen in cattle experiencing summer slump is also thought to be due to depressed prolactin levels (Thompson and Stuedemann 1993).

Fat necrosis is manifested by the presence of hard, mesenteric fat surrounding the intestinal tract from the abomasum to the rectum (Bush et al. 1979). These deposits can disrupt digestive processes and interfere with calving or renal function (Thompson and Stuedemann 1993) by causing inadequate space to be available for these functions in the abdominal cavity (Schmidt and Osborn 1993). As part of this syndrome, serum cholesterol concentration is reduced in cattle and sheep that are exposed to E+ fescue (Stuedemann et al. 1985). This has been interpreted as a response to stress and alters fat metabolism by increasing the saturated fatty acid concentration of fat deposits in steers grazing E+ tall fescue (Thompson and Stuedemann 1993).

Endophyte toxicity affects a variety of animal species. The greatest impact of endophyte-infected pastures is on beef cow-calf production as it is the largest livestock industry in areas seeded with E+ tall fescue (Hoveland 1986). The presence of endophyte has been correlated to decreased feed intake in livestock, especially when exposed to elevated temperatures (Hemken et al. 1981, Hemken et al. 1979). Vasoconstriction decreases an animal's ability to dissipate body heat through evaporative and non-evaporative mechanisms, which is further compounded by elevated ambient temperatures (Schmidt and Osborn 1993). As a result of hyperthermia, cattle seek shade and stand in water instead of grazing (Schmidt and Osborn 1993). An 8% depression in intake was seen in steers fed E+ hay which resulted in an average daily weight gain depression of 58% (Schmidt et al. 1982). Decreased palatibility of grass containing ergot alkaloids has also been suggested to contribute to an

animal's avoidance of E+ grass (Latch 1993). Ergot alkaloids can also have inhibitory effects on rumen motility by acting as dopamine agonists (Maas et al. 1982). This may affect assimilation of nutrients by reducing motility and absorption. When considering the effects of ergot alkaloids on weight gain in livestock, however, palatability and motility inhibition are believed to be of less importance than reduced feed intake (Thompson and Stuedemann 1993).

Beef cows with nursing calves were found to gain weight at a slower rate when placed on E+ feed than cows on E- feed (Schmidt et al. 1983). Their calves also had slower weight gains and reduced weaning weights compared with those on E- feed. Milk production is reduced by as much as 45% in beef cows (Schmidt et al. 1983) and 60% in dairy cows (Hemken et al. 1979) grazing E+ fescue. Milk fat percentage and total milk yield were lower in cows on E+ fescue, suggesting that a calf's nutritional milk value is substantially lower from cows on E+ feed (Brown et al. 1996). Heifers that had grazed E+ fescue as young calves had delayed puberty and decreased first-service pregnancy rates (Washburn et al. 1989). Pregnancy rates for cows on E+ fescue are also reduced (Schmidt and Osborn 1993).

The physiological effects of endophyte-infected fescue are more severe in mares and foals than in cattle (Schmidt and Osborn 1993). Endophyte toxicosis affects horses most significantly by increasing foal mortality and reproductive problems amongst mares (Hoveland 1993). Mares grazing E+ fescue during the last three months of gestation have substantial incidence of dystocia, agalactia, prolonged gestation and retained placentas (Monroe et al. 1988). Foals born to affected mares often have to contend with tough, thickened placentas and are weak and even stillborn at times (Thompson and Stuedemann 1993). An interesting difference between horses and cattle, however, is the lack of a carry-over effect when mares are exposed to E+ fescue and then changed to E- fescue (Schmidt and Osborn 1993). Mares exhibit a rapid turnover of the toxicants and overcome the negative effects of

E+ fescue to foal normally when they are removed from E+ fescue at least 30 days previous to foaling.

Losses to dairy cattle have been suggested to be minimal due to the high supplementation of these cattle with grain and low use of endophyte-infected tall fescue (Hoveland 1993). Wildlife may also suffer toxicity symptoms due to consumption of endophyte-infected tall fescue. These include rabbits whose population has declined in areas planted with tall fescue, mice who produce fewer young and have inadequate milk production when females were fed endophyte-infected seed (Zavos et al. 1988a), and deer who have been reported to exhibit staggers when consuming endophyte-infected perennial ryegrass (Mackintosh et al. 1982).

Laboratory mice have been shown to have a reduced reproductive capacity as determined through fertilization rates, pregnancy and litter size (Godfrey et al. 1994, Zavos et al. 1987a, Zavos et al. 1987b) and depressed growth of pups due to reduced suckling performance of the dam (Zavos et al. 1988a). Therefore, mice may serve as a model to detect genetic differences between resistant and susceptible animals (Hohenboken and Blodgett 1997). Recently, mice were selected as a model to determine whether exposure to ergot alkaloids could cause a measurable genetic divergence (Hohenboken et al. 2000, Wagner et al. 2000, Hohenboken and Blodgett 1997, Miller et al. 1994). The initial selection for divergence in response to tall fescue toxicosis was carried out for eight generations by Hohenboken and Blodgett (1997). Individual mice were tested for fescue toxicosis by quantifying the reduction in their growth rate when E+ fescue seed was present in the diet, compared to their growth rate when E- fescue seed composed the same proportion of the diet and genetically divergent lines were established.

Economic losses to the beef cattle industry due to E+ tall fescue were estimated to be $609 million per year in a national survey of 21 tall fescue-growing states with more than 25 million acres of tall fescue (Fribourg et al. 1991). A 20% decrease in calving rate correlates to a loss of about 885,000

calves. At an average 500-pound potential weaning weight and $0.80/pound sale weight, this amounts to an annual loss of $354 million. A decrease in weaning weight of 50 pounds per calf results in an annual loss of $255 million. Combined, this amounts to an annual loss of $609 million in the United States alone. This figure does not include losses from other livestock species, such as horses and sheep. The southeast United States is home to many horse farms where tall fescue is the primary pasture forage. Decreases in pregnancy rates and reproductive difficulties such as aborted fetuses can lead to large economic losses for a farm when considering the value of a single horse with a desirable pedigree.

Research on the toxic effects of endophytes in animals and potential solutions comprises one of the most important research problems in forage-livestock interactions (Hoveland 1993). Modification of the fungal endophyte to remove the harmful properties and maintain those which are beneficial to the plant has been suggested as the ultimate solution to the endophyte toxicity problem (Hoveland 1993). However, this would require replanting of millions of acres throughout the United States currently seeded with endophyte-infected tall fescue, a serious undertaking both in terms of labor and cost. A current goal in endophyte research is to identify biochemical parameters that differ between resistant and susceptible animals which could be applied to livestock to measure susceptibility amongst individual herds, breeds and species. Using the genetically divergent mice described previously compared to livestock permits the investigation of genetic biomarkers to fescue toxicosis utilizing less monetary resources and experimental time since mice reach reproductive maturity within two months, have a high fecundity, and environmental conditions can be easily controlled. Livestock could then be preferentially selected for breeding using these physiological biomarkers in order to confer greater resistance to endophyte toxicity within a herd.

Ergot alkaloids are used to treat a variety of human diseases, including depression of prolactin secretion in hyperprolactinemia and controlled

gonadotrophin secretion for Parkinson's disease with bromocryptine. Parkinson's disease has also been treated with the dopamine agonist dihydroergocryptine (Althaus et al. 2000). One of the available drugs used to treat migraine headaches is cafergot, a combination of caffeine and ergotamine tartrate (Dresser et al. 2000). A syndrome called 'St. Anthony's Fire' that appeared during the Middle Ages is thought to be due to ergotism and is associated with excessive ergotamine intake (Dresser et al. 2000) from grain infected with the fungus *Claviceps purpurea* (Peraica et al. 1999). Initial symptoms include edema in the legs with severe pain. If the disease is allowed to progress, gangrene can evolve with loss of the limb as the ultimate consequence of alkaloid ingestion. Although largely eradicated from the world, ergotism still appears sporadically in developing countries especially during times of drought and crisis. An epidemic was recorded in 1977-78 in Ethiopia where 47 people died from 93 total cases of ergotism due to infected wild oats (Demeke et al. 1979). Recently, a case of ergotism was found in an HIV-positive patient in Argentina who was administered ritonavir for his disease and self-administered ergotamine when experiencing migraine headaches (Vila et al. 2001). Ritonavir inhibited the P450 enzyme responsible for ergotamine metabolism, thus increasing the bioavailability ergotamine.

Hepatic Metabolism of Ergot Alkaloids

Ergot alkaloids undergo significant first-pass metabolism, particularly by the human cytochrome P450s CYP2D6 (N-6 dealkylation) and CYP3A4 (hydroxylation), and are primarily excreted via the bile with an elimination half life of 1-6 hours (Maurer et al. 1983). Early work showed that liver microsomes from rats induced with dexamethasone (a CYP3A inducer) showed a high incidence of hydroxylation on the tripeptide moiety of bromocryptine, suggesting that the tripeptide is essential for recognition by P450s (Maurer et al. 1983, Maurer et al. 1982). They also found that

bromocryptine was hydroxylated on the proline ring of the pseudo peptide moiety at C-8 (Maurer et al. 1983, Maurer et al. 1982). Longer incubations resulted in more extensive oxidation giving dihydroxylation products at C-8 and C-9 of the proline ring.

CQA 206-291 is a novel ergot alkaloid used in the treatment of Parkinson's disease. It contains a lysergic ring structure with a secondary amine off of C8 bonded to a sulfamide containing two ethyl groups. Research with CQA showed that CYP3A4 was the main human enzyme involved in its metabolism (Ball et al. 1992). This has clinical implications as CYP3A4 is the predominant P450 in human liver and is responsible for a wide variety of drug and xenobiotic metabolism. If CYP3A4 is induced or inhibited by coadministered compounds, the metabolism of ergot alkaloids would be altered as well. Ball et al. (1992) also found that the ergot alkaloids dihydroergotamine, bromocryptine and SDZ 208-911 were competitive inhibitors of metabolite formation, indicating that they are also metabolized by similar enzymes. Recently, the *in vitro* P450 metabolism of α-dihydroergocryptine was investigated in 11 human cell lines expressing distinct P450 isozymes (Althaus et al. 2000). It was found that CYP3A4 was the primary P450 isozyme responsible for oxidation of the alkaloid to mono- and dihydroxy metabolites.

Other evidence of CYP3A involvement in the metabolism of ergot alkaloids came from observations that co-administration of bromocryptine with erythromycin or troleandomycin resulted in inhibition of their metabolism (Peyronneau et al. 1994). Some macrolide antibiotics like erythromycin and troleandomycin inhibit CYP3A enzymes. Thus, co-administration of compounds dependent on CYP3A for metabolism are eliminated at a slower rate which increases their bioavailability. Experiments performed with other P450s showed that bromocryptine had little interaction with them, based on little or no difference spectra produced from rats treated with inducers of the CYP1A (3-methylcholanthrene), CYP2B (phenobarbital) and CYP4 (clofibrate) families (Peyronneau et al. 1994). Further, bromocryptine metabolism was

studied in liver microsomes from rats treated with phenobarbital and β-naphthoflavone (Moochhala et al. 1989). They found that metabolism of ethoxyresorufin, a marker of CYP1A activity, was inhibited in these groups of rats. They also found that bromocryptine bound to cytochrome P450s through a spectrophotometric assay, which suggests that it inhibits P450s by binding directly to the enzyme.

Recently, the drug cafergot (100 mg caffeine and 1 mg ergotamine tartrate) was given a new boxed warning by the FDA due to cases of serious and/or life-threatening peripheral ischemia associated with coadministration of cafergot with CYP3A4 inhibitors (personal communication, Timothy Duringer). Because CYP3A4 is the main enzyme in humans responsible for metabolism of cafergot, inhibition of this isozyme elevates serum levels of ergotamine. Thus, the risk for vasospasm leading to cerebral ischemia and/or ischemia to extremities is increased. Due to this increased risk, concomitant use of cafergot with CYP3A4 inhibitors is contraindicated.

To date, little is known about the hepatic metabolism and detoxification of ergot alkaloids in livestock. One group has done some preliminary investigation on the metabolism of ergot alkaloids in beef cattle and found that ergotamine was metabolized in a similar manner as the above rodent studies (Moubarak and Rosenkrans 2000). They found a metabolite profile for ergotamine in cattle liver microsomes that was similar to the profile exhibited by dexamethasone treated rats. The profile showed multiple metabolism products, which they suggested were hydroxylations of ergotamine. From this, they concluded that CYP3A may be responsible for metabolism of ergot alkaloids in beef cattle since dexamethasone is a CYP3A inducer in that species. They did not report identification of the metabolites produced from incubation of ergotamine in bovine liver microsomes with supporting mass spectroscopy or use of analytical standards, however, so their assumptions on the chemical identity, particularly on the site of hydroxylation, are purely speculative. This group continued their studies on ergotamine metabolism in

liver microsomes by incubating ergotamine with liver microsomes from dexamethasone-treated rats and compared the amount of ergotamine converted during incubation in these microsomes to rats treated with 10 or 100 nM dihydroergotamine, 10 or 100 nM ergonovine and corn oil control (Moubarak et al. 2003). They found no increase in 'CYP3A4 activity' (as determined by conversion of ergotamine to its metabolites in dexamethasone-treated rats) for rats treated with dihydroergotamine or ergonovine over control rats. However, no actual measurement of CYP3A4 protein concentration or its activity was reported.

Rumen Metabolism of Ergot Alkaloids

Little information is available on the bioavailability of ergot alkaloids in livestock that graze tall fescue (Stuedemann et al. 1998). This has led to some debate on which alkaloids are responsible for fescue toxicosis and if they are toxic in and of themselves or if they need to be bioactivated in the rumen/gastrointestinal tract or liver in livestock. One study examined the rate of appearance or clearance of ergot alkaloids via bile and urinary excretion from steers that were fed either E+ or E- tall fescue (Stuedemann et al. 1998). This study found excretion of lysergic acid-like compounds in the urine of steers on E+ feed and therefore implied that ergot alkaloids are hydrolyzed to lysergic acid moieties for absorption from the digestive tract and excretion into the urine. Approximately 20-fold more ergot alkaloids were excreted in the urine than in the bile from animals on E+ fescue. However, they quantified ergot alkaloid concentration with a competitive ELISA that is specific for compounds with a lysergic acid ring structure (Hill and Agee 1994). This assay has been proven to be ineffective in detecting ergopeptides with large peptide side groups, such as ergocryptine and ergotamine (Schnitzius et al. 2001). Thus, less polar, larger molecular weight compounds such as these ergopeptides will not be detected with this assay in bile or urine.

More recently, this group studied the transport of five ergot alkaloids or lysergic acid compounds across sheep ruminal gastric tissue (Hill et al. 2001). They surgically removed sheep reticular, ruminal and omasal tissues and placed them in parabiotic chambers. They applied 30.5 μM of lysergic acid, lysergol, ergonovine, ergotamine or ergocryptine to the mucosal side of the tissue and incubated the tissue at physiological conditions in a water bath for 240 minutes. They found that the rumen was capable of transporting 25% more alkaloids than the omasum and 600% more alkaloids than reticular tissues. In addition, the ergoline alkaloids (lysergic acid, lysergol and ergonovine) had a higher molar transport than the ergopeptides (ergotamine and ergocryptine).

Studies on the biodegradation of ergot alkaloids in rumen fluid are not as prevalent in the literature as those on ruminal transformation of pyrrolizidine alkaloids. This constitutes a current focus of research, however, as understanding ergot alkaloid metabolism in the rumen and what role bioactivated and/or detoxified molecules from the rumen play in fescue toxicosis is important in formulating strategies to combat this disease. Sheep appear to be more resistant to endophyte toxicity than cattle, based on levels estimated to be toxic for these species (200 ppb for cattle (Rottinghaus et al. 1991) and 500-1000 ppb for sheep (Debassai et al. 1993)). As with PAs, this difference in toxicity could be attributed to a combination of factors with differences in rumen metabolism playing a potentially important role.

Biodegradation of ergovaline and ergonovine have been shown in an *in vitro* assay with ruminal fluid from a steer previously fed tall fescue (Moyer et al. 1993). Nearly 100% of the soluble ergovaline was metabolized within 48 hours. The degradation of the pyrrolizidine alkaloids N-formyl loline and N-acetyl loline were also investigated as they make up a portion of the alkaloids found in tall fescue (Westendorf et al. 1993). After 48 hours, N-formyl loline was degraded to a greater extent than N-acetyl loline when incubated with rumen fluid from sheep on an E+ diet. They found that degradation increased

over the 48 hour incubation, with the majority of loline degradation occurring in the second 24 hours. This is probably due to a lag phase whereby microbes need to adapt to new energy sources before rapid degradation occurs. In addition, *in vitro* experiments with bovine ruminal fluid have shown biodegradation of inoculated ergovaline over 48 hours (Craig 1995). Recent research has suggested a role for ruminal adaptation and consequent detoxification of ergovaline in sheep rumen fluid (Tor-Agbidye et al. 2001).

In vitro degradation of E+ tall fescue was measured in bovine rumen fluid over 48 hours with the ergoline ELISA assay mentioned above (Stuedemann et al. 1998). Samples consisted of rumen fluid and 0.4 g E+ tall fescue. Ergoline alkaloids were measured at 0, 12, 24 and 48 hours in both the pellet (E+ fescue plant material) and supernatant. Over 48 hours, it was found that the pellet decreased in ergoline alkaloid concentration while the supernatant increased. The total amount of alkaloids did not change over the 48 hour period. This suggests that the ruminal microbes liberated the alkaloids from the plant tissue and that the lysergic ring remained intact during the digestive process. Further, the amount of ergoline alkaloids present in the supernatant paralleled that in the urine. Given the caveats mentioned above with this assay, however, this only traces the fate of the ergoline alkaloids. The metabolic fate of all ergot alkaloids, including both ergopeptide and ergoline alkaloid groups, remains to be investigated.

Thesis Overview

Species susceptibility to toxicity is a complex interaction involving species specific differences in both pre- and post-absorption metabolism. In the case of pyrrolizidine alkaloids, sheep have been observed to be resistant to toxicity while cattle are susceptible. Previous studies have categorized the mechanism of resistance to PA toxicity in ruminants into two theories: 1) that the microbial population of the rumen of resistant species can more efficiently

eliminate PAs from ingested plants or 2) that the hepatic metabolism of PAs in resistant species produces a higher ratio of non-toxic to toxic metabolites. Research in this laboratory has focused on the mechanism of pyrrolizidine alkaloid metabolism in rumen fluid and isolated bacterial cultures from sheep and cattle. Detoxification of PAs from *Senecio jacobaea* has been demonstrated in rumen fluid from sheep and cattle (Craig et al. 1992a, Wachenheim et al. 1992a, Wachenheim et al. 1992b). Sheep rumen fluid degrades PAs within 2-6 hours, while bovine rumen fluid requires 24-48 hours to degrade the same concentration of PAs. In addition, sheep rumen fluid contains a larger number of PA-degrading bacteria than cattle rumen fluid, which suggests that the lower number and decreased metabolic efficiency of bacteria in the bovine rumen is a factor in their susceptibility to PA toxicity.

In an attempt to establish the relative contribution of hepatic metabolism of pyrrolizidine alkaloids in sheep and cattle, a side-by-side comparison of the *in vitro* hepatic metabolism of senecionine in sheep and cattle liver microsomes was conducted in Chapter 2. Senecionine is one of the pyrrolizidine alkaloids found in *Senecio jacobaea*, a plant that is ubiquitous in rangelands of the Pacific northwest and a major cause of hepatotoxicity in livestock there (Craig et al. 1991). Thus, it was chosen as the model PA for these metabolism studies. Evidence for the theory that hepatic metabolism differences lead to differing susceptibilities in PA toxicity has centered on the relative amount of DHP produced during metabolism of PAs (Cheeke 1994). However, results in this work on DHP formation mirrored previous findings which suggested that there is not a strong correlation between *in vitro* DHP formation from liver microsomes and a species' susceptibility to PA toxicity (Huan et al. 1998a). Some differences were seen between the species during senecionine metabolism. Sheep metabolized a larger amount of the parent compound and had a higher rate of senecionine *N*-oxide formation compared to cattle. This could aid sheep in eliminating a potentially toxic compound through a relatively nontoxic pathway faster than cattle who form DHP at a much higher rate

relative to *N*-oxide. The kinetics of DHP and senecionine *N*-oxide formation in sheep and cattle were also studied in Chapter 2.

Cytochrome P450s, FMOs and carboxylesterases seem to play similar roles in the metabolism of PAs in sheep and cattle as determined through the use of chemical and immunochemical inhibitors. CYP3A appears to play a larger role in DHP formation in cattle than in sheep. The effect of glutathione on PA metabolism was also investigated through addition of glutathione and/or cytosol to microsomal incubations. This study supports the results of previous research which found that glutathione conjugation plays a significant role in the detoxification of PAs for both sheep and cattle (Huan et al. 1998a, Yan and Huxtable 1995, Reed et al. 1992). Given the overall similarities found in this study, differences in susceptibility to PA toxicity cannot be solely attributed to differences in liver metabolism.

Experiments investigating differences in PA metabolism between genders of ruminant species are not very prevalent in the literature. Given that gender differences in PA metabolism of livestock could ultimately affect management practices, one of the objectives in Chapter 2 was to determine if differences existed in the hepatic metabolism of senecionine between male and female sheep and between male and female cattle. Due to the marginally significant differences found in *N*-oxide formation in cattle and kinetic measurements in sheep, further studies with a larger sample size are needed to determine if these differences are due to gender-specific influences on monooxygenase presence or activity.

Research has indicated that there is a relationship between diet, bacterial status and hepatic cytochrome P450 content in rats (Nugon-Bandon et al. 1998). This suggests that differential rumen microbial compositions of sheep and cattle are linked to the capabilities of the liver to activate or detoxify PAs. Combined with the findings in this study, it appears that species susceptibility is a complex interaction involving animal and possibly gender-specific

differences in both pre- and post-absorption metabolism (Stegelmeier et al. 1999).

Tall fescue (*Festuca arundinacea*) plants infected with the endophyte *Neotyphodium coenophialum* produce ergot alkaloids which are toxic to livestock that consume endophyte-infected grass. Ergovaline is the most predominant ergot alkaloid found in tall fescue and so is commonly referred to as the alkaloid responsible for fescue toxicosis (Yates and Powell 1988, Lyons et al. 1986). The current method for testing grass samples for the presence of ergot alkaloids is via HPLC detection of ergovaline. This procedure is both costly and time-consuming and requires the use of sophisticated analytical equipment and personnel. A rapid and inexpensive assay such as ELISA would aid in analyzing a larger number of samples and would decrease the cost of analysis for these toxins. Details of a comparison between a competitive ELISA assay developed with a monoclonal antibody specific for the lysergic ring structure of ergot alkaloids and the ergovaline HPLC are discussed in Chapter 3. Since both procedures test for structurally distinct compounds (HPLC tests for ergovaline only and ELISA tests for ergoline alkaloids), no correlation was evident between the two assays. Thus, each method is a valid indicator of toxicity for the specific toxin(s) they measure. In addition, clarified rumen fluid was tested as a matrix for use in the ELISA assay to determine if the ELISA could be used as a screening tool in livestock metabolism experiments. The ELISA exhibited similar variation when standards made up in clarified rumen fluid were used. Thus, the assay was determined to be adaptable to other matrices.

Laboratory mice may serve as a model to detect genetic differences between resistant and susceptible animals since they have been shown to have reduced reproductive capacity when exposed to an endophyte-infected diet (Godfrey et al. 1994, Zavos et al. 1988b, Zavos et al. 1987a, Zavos et al. 1987b). Using mice compared to livestock permits the investigation of genetic selection of sensitivity to fescue toxicosis utilizing less monetary resources

and experimental time. Chapter 4 investigated if the genetic divergence exhibited previously in mice selected for susceptibility or resistance to fescue toxicity had persisted over ten generations of relaxed selection. Experimental groups were endophyte-susceptible and endophyte-resistant mouse lines containing males and females placed on an endophyte-free diet or challenged with and endophyte-infected diet over a two week period. Average daily gain of mouse pups and metabolism of the ergot alkaloid ergotamine in mouse liver microsomes were compared between lines and genders. No differences in weight gain of pups between the lines remained. Hepatic metabolism in mouse liver microsomes presented a more complicated picture. Females exhibited a larger amount of transformation products 1 (M1 and $M1_i$) and 2 (M2 and $M2_i$) than males in the endophyte-free group. Animals on the endophyte-infected diet showed increased amounts of polar metabolites 3 and 4 as compared to animals on the E- diet. Analysis of ergotamine, metabolites 1, 2 and their isomers was performed by LC-MS. The identity of ergotamine and its isomer in addition to four hydroxylation products was confirmed.

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Chapter 2

COMPARISON OF HEPATIC *IN VITRO* METABOLISM OF THE PYRROLIZIDINE ALKALOID SENECIONINE IN SHEEP AND CATTLE

Jennifer M. Duringer and A. Morrie Craig

Abstract

Pyrrolizidine alkaloids (PAs) are among the most prolific group of plant toxins affecting livestock, wildlife and human health throughout the world. Significant variability exists in ruminant species susceptibility to toxicity, which has been attributed to either differences in the metabolism of PAs by ruminal microbes or differences in hepatic bioactivation and detoxification of PAs. In this study the *in vitro* hepatic metabolism of senecionine in sheep (resistant) and cattle (susceptible) of both genders was compared using liver microsomes and cytosol. The concentration of senecionine, senecionine *N*-oxide and 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) were measured by HPLC. Sheep formed more *N*-oxide than cattle. This affected the DHP/*N*-oxide ratio which was larger in cattle. Senecionine bioactivation to DHP did not differ between the species. Sheep also metabolized more of the parent compound senecionine than cattle. The kinetic parameters K_m and V_{max} for DHP and senecionine *N*-oxide (*N*-oxide) formation were defined for both species and genders. Sheep had a higher V_{max} and K_m for *N*-oxide than cattle. Gender differences were apparent in the formation of *N*-oxide between female and male cattle and the formation of DHP between female and male sheep. Female cattle produced more *N*-oxide which decreased their DHP/*N*-oxide ratio in comparison to male cattle. Male sheep produced more DHP than female sheep did. Female sheep had higher K_m and V_{max} values for both DHP and *N*-oxide than male sheep.

Through the use of chemical inhibitors, it appears that the cytochrome P450 CYP3A plays a larger role in the metabolism of SN to DHP in cattle than in sheep. The activity of CYP3A and CYP2B as measured by 6β- and 16β-hydroxylation of testosterone were similar in both species and genders. Cattle also had more CYP3A protein than sheep as measured by Western blotting. Similarities exhibited by both species include a large influence from P450s on DHP formation and from flavin-containing monooxygenases on *N*-oxide formation. A decrease in DHP formation was evident for both species when

adequate glutathione was present. Surprisingly, a decrease in *N*-oxide formation was seen with the addition of cytosol to *in vitro* incubations in the GSH experiments, perhaps due to additional detoxifying components in the cytosol. In summary, the sheep's ability to form more *N*-oxide relative to DHP and to metabolize more of the parent compound combined with glutathione conjugation may partially explain their resistance to PA toxicity. Cattle formed more DHP relative to *N*-oxide and had a slower rate of elimination of the parent PA which may partially explain their susceptibility to PA toxicity.

Introduction

More than 660 pyrrolizidine alkaloids (PAs) have been isolated from over 6000 plant species throughout the world (Xia et al. 2003). It is estimated that 3% of all flowering plants contain toxic PAs (Logie et al. 1994, Smith and Culvenor 1981). PAs have a widespread geographic distribution and cause toxicity problems in livestock across all continents. In the northwest United States, tansy ragwort (*Senecio jacobaea*) has been described as the major toxic plant causing livestock loss (Craig et al. 1991). Losses induced by plant poisonings can be direct or indirect. Direct losses are measured in annual mortality attributed to poisonous plants and have been estimated to be $20 million in Oregon alone (Craig et al. 1991). Indirect losses include the cost of control measures (e.g. fencing and veterinary expenses), temporary or permanent non-utilization of toxic pastures and the diminished value of infested land (Kellerman et al. 1996). South Africa is also affected by PA-containing plants where *seneciosis* (toxicity caused by plants containing the PA senecionine) is the third most important cause of poisoning in livestock (Kellerman et al. 1996). Seneciosis accounts for 10% of cattle and 5% of small stock (such as sheep and goats) mortality due to plant poisonings. In a third world country with a struggling economy such as South Africa, these mortalities have a tremendous impact on farmers who depend on healthy

livestock for food, clothing and as a means of transport (Christo Botha, personal communication).

PAs are largely hepatotoxins, capable of causing both acute and chronic toxicities (Mattocks 1986c). Acute toxicity rarely occurs under natural conditions. It usually requires large doses of PAs, such as when PAs are rapidly absorbed by intraperitoneal or intravenous injection, and results in severe hemorrhagic necrosis of the liver and rapid death. Chronic toxicity usually results in the development of veno-occlusive disease of the liver which occludes small branches of the hepatic veins and causes ascites, edema, reduced urinary output and can lead to cirrhosis and death. PAs can also affect extrahepatic organs such as the lungs and heart as well as initiate cancer development in a variety of animal models (Prakash et al. 1999, Mattocks 1986a, Mattocks 1986b).

Susceptibility to PA toxicity is influenced by many factors including species, age and sex as well as other temporary factors such as the mode of PA administration, biochemical, physiological and nutritional status (Stegelmeier et al. 1999). The animal species resistant to PA toxicity include sheep, guinea pigs, rabbits, hamsters, gerbils and Japanese quail, whereas rats, cattle, horses and chickens are susceptible to PA toxicity (Huan et al. 1998a). Cattle and horses develop chronic terminal hepatic disease after consuming as little as 5% of their body weight in PA-containing plant material, while sheep are able to ingest up to 300% of their body weight without adverse effects (Craig et al. 1991, Cheeke 1984, Culvenor 1980). This has resulted in considerable debate in the literature on whether the relative resistance of sheep to PA toxicity compared to more susceptible species such as cattle is due to differences in the microbial population of the rumen or to differences in hepatic metabolism of these compounds. The substantial interspecies variability in the toxicological response to PAs makes extrapolation of data from animal studies of one species to another difficult.

To test differences in hepatic metabolism of the PAs in sheep and cattle, a study placed an indwelling portal vein catheter surgically in order to isolate the liver and bypass the rumen. Once the liver was isolated, PA extracts from the plant *Senecio jacobaea* at levels similar to those expected in the animal diet were infused via the catheter (Craig et al. 1987, Craig et al. 1986, Craig et al. 1985). Sheep and cattle developed similar clinical, enzymatic and histopathological changes in the liver from both acute and chronic exposure to PAs, suggesting that the protective mechanism of sheep is derived from their gastrointestinal system. Thus, attention was focused on the possibility that ruminal microbes in sheep can degrade the alkaloids before they enter systemic circulation as bioactivated toxins.

Pyrrolizidine alkaloid degrading microbes have been isolated from the rumen contents of both sheep and cattle. The PAs from the plant *Helioptropium europaeum* studied *in vitro* and *in vivo* in sheep ruminal fluid were converted to non-toxic 1-methylene and 1-methyl derivatives (Lanigan 1970, Lanigan and Smith 1970, Dick et al. 1963). This led to the isolation of *Peptostreptococcus heliotrinreducens* from sheep rumen contents (Lanigan 1976) which was identified as the bacterium responsible for PA resistance. Rumen fluid from both sheep and cattle maintained on a grass/alfalfa diet containing no PAs exhibited detoxification of PAs from *Senecio jacobaea* including senecionine (Wachenheim et al. 1992b). It was found that ovine ruminal fluid degraded PAs within 2-6 hours, while bovine ruminal fluid required 24-48 hours. In addition, ovine rumen fluid had 3.0×10^7 PA-degrading bacteria/ml while bovine rumen fluid had 1.1×10^7 PA-degrading bacteria/ml when counted by most probable number analysis. This suggests that susceptibility to PA toxicity may be due to the lower bacteria concentration which decreases the rate of detoxification transformations in the bovine rumen. Therefore this may be a factor that influences absorption and/or bioactivation of PA toxins. A feeding trial demonstrated that cattle are protected from PA toxicity after transfer of ovine ruminal fluid from sheep

eating *S. jacobaea* into the rumen of cattle (Johnston et al. 1998). Thus, the use of a probiotic as a management tool in protecting susceptible livestock has been proposed. Further research has been done in this area to enrich and identify the microbes responsible for PA detoxification (Craig 1995), as well as the pathway of bacterial metabolism of these compounds (Hovermale and Craig 2002, Hovermale 1998). A mixed culture of anaerobic microbes from sheep rumen fluid that metabolizes macrocyclic PAs (L4M2) was studied to identify the metabolites formed from inoculation with monoester, diester and macrocyclic PAs (Hovermale and Craig 2002). L4M2 formed 1-methylene compounds as intermediates or end-products for all compounds tested.

Metabolism of PAs occurs by three main pathways in the liver (Mattocks 1986c). PAs can be oxidized to *N*-oxides by the Phase I cytochrome P450 and/or flavin-containing monooxygenase (FMO) enzymes (Figure 2.1). PAs can be hydrolyzed to necine base and necic acid moieties via carboxylesterases. Or the PA can be oxidized by cytochrome P450 (P450) monooxygenases to reactive carbonium ion intermediates which are unstable and either undergo rapid hydrolysis to DHP and/or covalently bind to DNA or other cellular nucleophiles. The pyrroles have been shown to react with glutathione (GSH) to form a DHP-GSH conjugate (Reed et al. 1992) which increases the water solubility of the pyrrole. The conjugate is subsequently excreted into the bile or sinusoidal blood and ultimately into the urine (Yan and Huxtable 1995). Alternatively, the soluble DHP-GSH conjugate can serve as a transport vehicle to other organs such as the lung, in which toxicity can be elicited if the pyrrole is released from GSH (Cheeke and Huan 1995).

Evidence that hepatic metabolism differences lead to differing susceptibilities in PA toxicity among species has historically centered on the relative amount of DHP produced during metabolism of PAs. In general, resistant species such as guinea pigs, sheep and quail are said to have low rates of DHP formation while susceptible species such as horses and cattle

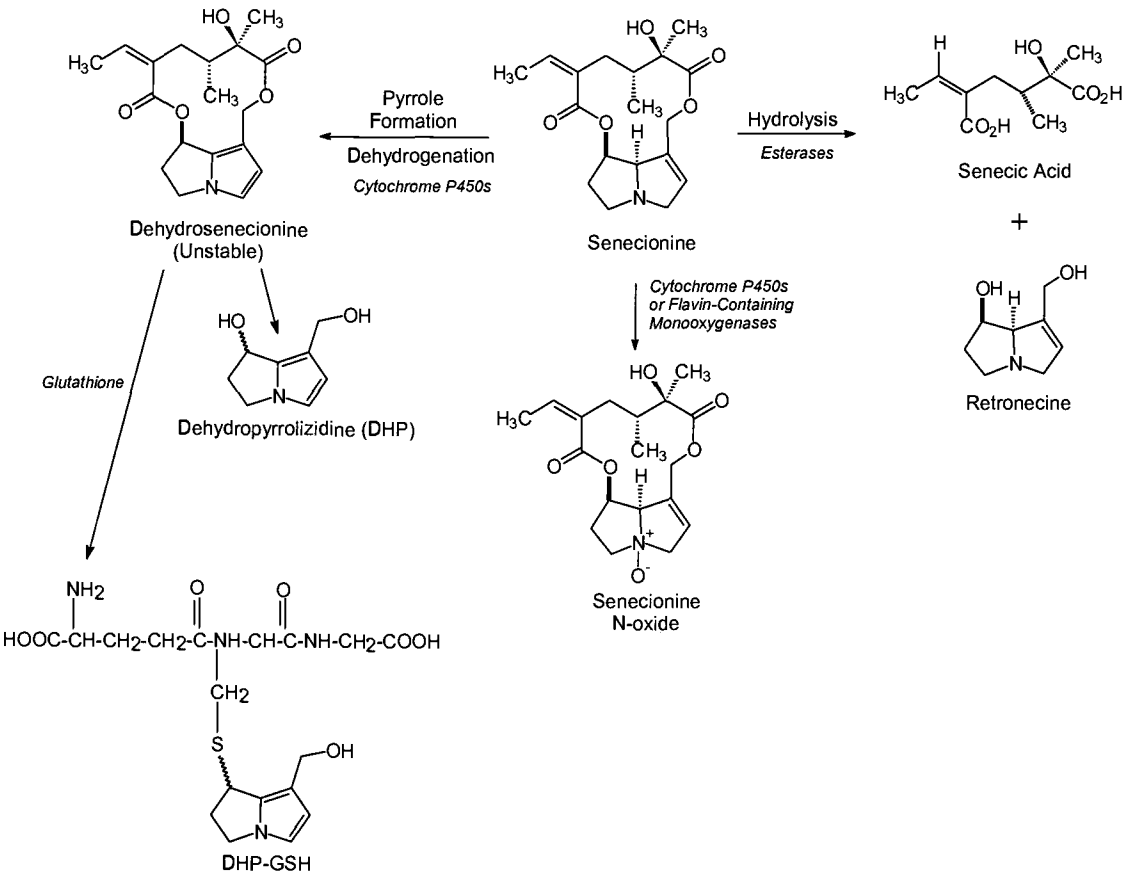

Figure 2.1 Major pathways of hepatic PA metabolism.

have higher rates of DHP formation (Cheeke 1994). This suggests that PAs are bioactivated at a slower rate in resistant species. Thus, identification of the specific enzymes that are involved in biotransformation of PAs to DHP may be an important step in understanding the potential hazards of PAs in animals and humans (Huan et al. 1998b).

Several studies have looked at individual species in order to investigate which of the metabolic pathways confers resistance or susceptibility to PA toxicity. Metabolism of senecionine in liver microsomes from rats, rabbits and guinea pigs were studied using identical incubation conditions (Miranda et al. 1992). The DHP/*N*-oxide ratios were found to be 0.57, 2.23 and 0.21 for rat, rabbit and guinea pigs, respectively. Based on the high amount of DHP

formed relative to *N*-oxide, it would appear that rabbits are most susceptible to PA toxicity, followed by rats and guinea pigs. This is not the case, however, as rats are generally more susceptible to PA toxicity than guinea pigs and rabbits (Mattocks 1986c). In the guinea pig, it was found that resistance to senecionine toxicity is due to low DHP formation and high *N*-oxide formation carried out by FMOs (Miranda et al. 1991b) and CYP2B (Chung et al. 1995). Huan et al. (1998b) compared the production of DHP from senecionine amongst sheep and hamsters (resistant species), and found no strong correlation between DHP formation and an animal's susceptibility to PA toxicity. They found that DHP formation was catalyzed mainly by P450s, while SN *N*-oxide was formed mainly by FMOs. Sheep also utilized carboxylesterases more so than hamsters but both species showed a high rate of DHP-GSH conjugation. Thus, they concluded that species differences appeared to be dependent on hepatic microsomal enzymes and their catalytic capability. In addition, resistant species can abate PA toxicity by conjugating DHP to GSH (Chung and Buhler 1995), eliminating the parent compound at a faster rate than susceptible species (Huan et al. 1998a) and absorbing more of the PA into the enterocytes of the small intestine where it is metabolized by P450s to toxic metabolites which alkylate cellular macromolecules. However, since the enterocytes slough off every few days, the PAs would be subsequently excreted into the feces and eliminated from the animal.

To help resolve the debate on how resistant species counter PA toxicity, side-by-side *in vitro* metabolic studies on the bioactivation and detoxification pathways with sheep (resistant) and cattle (susceptible) liver microsomes were carried out to determine the fate of PAs in each species. DHP and *N*-oxide formation were monitored by HPLC as well as the amount of senecionine remaining after incubation. Chemical and immunological inhibitors were used as indirect methods to determine the relative contribution of P450s, FMOs and carboxylesterases in PA metabolism. Western blots measured the amount of specific P450 and FMO isoenzymes, while other assays were performed to

determine the activity of these enzymes. The addition of glutathione to determine its effects on metabolite formation was studied as well. The purpose of these experiments was to characterize the specific mechanisms of hepatic mixed-function oxidase activation and detoxification of PAs in sheep and cattle of both genders to determine if the difference in toxicity susceptibility of these two species is due in part to differences in metabolic capabilities of the liver using senecionine as the model PA. Genders of both species were studied to determine if a gender difference in the metabolism of PAs exists.

Materials and Methods

Chemicals and antibodies

Senecionine (purified from extracts of *Senecio triangularis*) was generously provided by Dr. J.N. Roitman (USDA-ARS, Western Regional Research Center, Albany, CA) and was also purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). Synthetic standards of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) and SN *N*-oxide were prepared as described in Kedzierski and Buhler (1986). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide diphosphate (NADPH), glutathione, SKF-525A, methimazole, phenylmethyl sulfonyl fluoride (PMSF), thiourea, trizma base, ethylenediamine tertraacetic acid (EDTA), glycerol, Triton X-100, dithiothreitol (DTT), 1-chloro-2,4-dinitrobenzene (CDNB), bovine serum albumin, mono- and dibasic potassium phosphate, troleandomycin (TAO), 5,5'-dithio-(2-nitrobenzoic acid) (TNB), *N*-ethylmaleimide, sodium phosphate, sodium dodceyl sulfate, glycine and pyronin Y were obtained from Sigma (St. Louis, MO). Coomassie Reagent and preimmune rabbit IgG were purchased from Pierce (Rockford, IL). Potassium chloride, acetic acid and sodium chloride were purchased from Fisher Chemical Company (Fairlawn, NJ). Tri-*o*-cresyl phosphate (TOCP)

was purchased from Chem Service (West Chester, PA). Dimethylsulfoxide (DMSO) was obtained from Cambridge Isotope Laboratories (Andover, MA). Formamide was purchased from ICN Biochemicals, Inc. (Aurora, OH). [^{14}C]-Testosterone was purchased from NEN (Boston, MA). 6β- and 16β-Hydroxytestosterone were purchased from Steraloids (Newport, RI). Thirty percent acrylamide (29:1), ammonium persulfate, TEMED (N,N,N,N'-tetra-methylethylenediamine, β-mercaptoethanol and Tween-20 were obtained from BioRad (Hercules, CA). Carnation milk powder was from Nestle (Glendale, CA). Goat polyclonal antibody raised against rat NADPH cytochrome P450 reductase, mouse monoclonal antibody raised against human CYP3A4/5/7, rabbit whole serum antibody raised against human FMO1, rabbit whole serum antibody raised against human FMO3 and rabbit whole serum antibody raised against human FMO5 antibody kits were obtained from BD Gentest (Bedford, MA). If standard was not included in the kit, it was purchased from BD Gentest at the time the antibody was ordered. Rabbit polyclonal antibody raised against rat CYP2B1/2 was purchased from Xenotech (Leneza,KS). ECL protein molecular weight markers, the ECL detection kit reagents and Hyperfilm were purchased from Amersham (Piscataway, NJ). Solvents were HPLC grade and were obtained from Mallinckrodt (Paris, KY). Water was obtained from a Millipore MilliQ purification system.

HPLC system

A Waters (Milford, MA) 2690 Separations Module with a Waters Photodiode Array Detector was used for the HPLC analyses in this study.

Animals and liver preparation

Twenty grams of liver tissue from multiple locations in the liver were collected immediately after slaughter from four adult male sheep, four adult female sheep, four adult male cattle and four adult female cattle (for a total of 16 animals) at a commercial slaughterhouse at Oregon State University. The tissues were immediately rinsed in ice-cold 1.15% KCl buffer and then placed in liquid nitrogen and pulverized in a Robot Coupe Blixer RSI BX6 blender

(Ridgeland, MS). They were then homogenized following a modification of the protocol from Huan et al. (1998a). Ten grams of pulverized liver tissue were placed in 40 ml of homogenization buffer (final concentration of 0.1M Tris base (pH 7.5), 0.1M KCl, 1mM EDTA, 1 mM DTT, 0.1 mM PMSF) and homogenized in an ice bath using a Potter-Elvjhem homogenizer. The suspension was centrifuged at 10,000*g* for 30 minutes at 4°C. The supernatant was centrifuged at 105,000*g* for 60 minutes and the centrifuged supernatant was saved as the cytosol fraction. The microsomal pellet was washed, resuspended in homogenization buffer and centrifuged at 105,000*g* for an additional 60 minutes. The microsomal pellet was resuspended in 10 ml buffer with a final concentration of 10 mM Tris acetate (pH 7.4), 0.1 mM EDTA, 20% glycerol, 1 mM DTT and 0.1 mM PMSF using a Potter-Elvjhem homogenizer maintained in an ice bath. Aliquots of the microsomal mixture were stored at -80°C until used.

Protein concentration determination

Microsomal and cytosolic protein content were determined with bovine serum albumin as the standard as described previously (Lowry et al. 1951). Microsomes were diluted 1:100 in H_2O. A volume of 100 µl of the diluted sample, standard (50, 100, 200 and 300 µg/ml bovine serum albumin) or blank (H_2O) were added to test tubes in triplicate. One ml of Coomassie Reagent was added to the test tubes and mixed with a vortex mixer. Into a 96-well plate was dispensed 200 µl of each reaction mixture. The absorbance was measured at 595 nm using a spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA). The protein concentration was calculated from the standard curve produced from the bovine serum albumin standards.

Cytochrome P450 concentration determination

Total cytochrome P450 concentration was estimated by modification of a previously reported method (Omura and Sato 1964). All steps were carried out in ice until measured in the spectrophotometer. Microsomes were diluted in buffer (final concentration of 0.1M potsssium phosphate (pH 7.4), 1 mM

EDTA and 20% glycerol) to a protein concentration of 2.0 mg/ml. The sample solution was saturated with carbon monoxide by bubbling gas through the liquid for 1 minute at a rate of ~1 bubble/second. The sample was then divided between a sample and reference cuvette. A dual beam spectrophotometer was set to scan a wavelength range of 400-500 nm. A baseline was recorded on the spectrophotometer in this range. The sample cuvette was removed and a few milligrams of sodium dithionite were added. The cuvette was covered in parafilm and mixed thoroughly. The sample cuvette was placed back in the spectrophotometer and a scan from 400-500 nm was recorded. This scan was repeated until it was reproducible (until the trace did not vary from the previous attempt). The amount of cytochrome P450 was calculated by measuring the difference in absorbance between 480 nm and 450 nm using an extinction coefficient of 106 $mM^{-1}cm^{-1}$.

In vitro *metabolism of senecionine by microsomes*

The basic microsomal incubation consisted of 0.5 mg protein, 0.1 M potassium phosphate (pH 7.6 for most studies, pH 8.5 for FMOs), 1.0 mM EDTA, and an NADPH-generating system (final concentration of 10.0 mM glucose-6-phosphate, 1.0 unit/ml glucose-6-phosphate dehydrogenase and 1.0 mM NADPH) in a total volume of 0.5 ml. The mixture was equilibrated for 5 minutes at 37°C in a shaking metabolic incubator at 100 cycles/minute. The reaction was initiated with addition of 0.5 mM senecionine (or 0.05-1 mM for kinetic incubations) and incubated for 60 minutes. The reaction was terminated by rapid cooling in ice water. The reaction mixture was then vortexed and centrifuged at 15,000*g* for 30 minutes at 4°C. An aliquot of the supernatant was analyzed by HPLC for DHP and senecionine *N*-oxide with a PRP-1 column (150 x 4.1 mm, 10 µm pore size from Hamilton (Reno, NV)) at λ = 220 nm on the HPLC system as modified from a previous study (Kedzierski and Buhler 1986). The gradient was run with acetonitrile (A) and 0.01 N NH_4OH (B) prepared fresh daily in the following steps:

Time	Flow rate (ml/min)	A	B
0	1.0	0.5%	99.5%
5	1.0	10%	90%
15	1.0	40%	60%
30	1.0	40%	60%
40	1.0	0.5%	99.5%
45	1.0	0.5%	99.5%

Data was captured by a computer with Millenium software (Waters). DHP, senecionine *N*-oxide and senecionine were identified and quantified by comparison with co-eluting standards. Incubations containing no NADPH were run as negative controls.

Chemical and immunochemical inhibitors

When chemical inhibitors (0.5 mM SKF-525A, 0.25 mM methimazole, 1.0 mM PMSF and 0.1 mM TOCP in DMSO, 10 µM TAO in acetone, 0.25 mM thiourea in H_2O) were used, the microsomal mixture was preincubated for 20 minutes at 37°C with inhibitor before addition of senecionine. Control incubations contained no NADPH or an equal volume of DMSO or acetone. Concentration of immunochemical inhibitors was 2.5, 5 and 10 mg IgG/nmol total P450 for antibodies raised against P450 and FMO proteins and 10, 20 and 30 mg IgG/nmol total P450 for the antibody raised against NADPH cytochrome P450 reductase. A total of 0.1 nmol P450 was used in the incubations from sheep and cattle. Microsomes were preincubated with antibody for 20 minutes at room temperature before addition of buffer and NADPH-generating system and senecionine and were carried out as described above. Control incubations had an equivalent amount of preimmune rabbit IgG.

Role of GSH in the metabolism of SN

Microsomal incubations were carried out with the reaction components as detailed above in the *in vitro* metabolism of senecionine, in addition to 2.0 mM GSH, 100 µl cytosol, or 2.0 mM GSH and 100 µl cytosol. The samples were

analyzed for DHP and *N*-oxide as described and compared to control incubations containing no GSH or cytosol.

GSH concentration was measured using a previously reported protocol (Hissin and Hilf 1976). Twenty-five microliters of cytosol were diluted in 225 µl 0.1 M sodium phosphate/5 mM EDTA buffer. Ten microliters of cytosol and 180 µl buffer was added to the well of a 96-well plate. GSH standards of 16.3, 32.5, 97.6 and 162.5 pmol and a blank (buffer) were added to the plate as well. Ten µl of a 1 mg/ml o-phthalaldehyde in methanol solution was added to all wells. The plate was incubated at room temperature for 15 minutes and then read on a spectrofluorometric plate reader (Molecular Devices Rockford, IL) at λ_{em} = 420 nm and λ_{ex} = 350 nm. Samples were calculated from a standard curve generated with the GSH standards. Average final GSH concentrations from 100 µl cytosol in the 0.5 ml incubation mixture were 0.02 mM for female cattle, 0.02 mM for male cattle, 0.03 mM for female sheep and 0.02 mM for male sheep.

Glutathione-S-transferase (GST) was measured according to a previously reported method (Habig et al. 1974). A volume of 50 µl CDNB, 20-100 µl microsomes and 0.1 M potassium phosphate buffer (pH 6.5) were added to a reaction vessel for a final volume of 1 ml. One microliter of Triton X-100 and 100 µl of 10 mM glutathione were added to start the reaction. The change in absorbance at λ = 340 nm was recorded on a spectrophotometer for three minutes. An extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ was used to calculate GST in the samples.

Methimazole-enhanced TNB oxidation

To measure FMO activity, methimazole-enhanced oxidation of TNB was carried out following a modification of a previous method (Dixit and Roche 1984). Buffer (final concentration of 100 mM tricine/1.0 mM EDTA (pH 8.4)) which had been aerated for 30 minutes was added to two cuvettes in addition to 0.5 mg microsomal protein, H_2O and 0.1 mM NADPH to 500 µl final volume

and incubated at room temperature for 40 seconds. *N*-Ethylmaleimide (25 µM) in ultrapure formamide was added to inhibit non-FMO activity and incubated for 40 seconds at room temperature. DTT (25 µM) and 60 µM TNB were added and incubated for 40 seconds at room temperature. Twenty microliters H_2O were added to the reference cuvette and both cuvettes were incubated for 3 minutes at 37°C in a shaking metabolic incubator. Methimazole (1 mM) was added to the sample cuvette, mixed and read at λ = 412 nm for 4 minutes. The change in absorbance was used with an extinction coefficient of 28.2 mM^{-1} cm^{-1} (pH 8.4) for TNB oxidation to calculate nmol methimazole-enhanced TNB-oxidation/min^{-1}/mg^{-1}.

Testosterone 6β- and 16β-hydroxylation

Total P450 (0.05 nmol), 0.1M sodium phosphate buffer (pH 7.4), 0.5 mM EDTA and milliQ H_2O were added to a reaction vessel for a final volume of 0.5 ml as described previously (Pearce et al. 1996). [^{14}C]-Testosterone (10 µCi/µmol) was added to the reaction vessel for a final concentration of 100 µM testosterone. It was allowed to preincubate for 3 minutes at 30°C on a shaking incubator. The reaction was initiated by addition of 0.5 mM NADPH and incubated for one hour at 30°C. The reaction was stopped using 3 ml of methylene chloride and centrifuged at 2000*g* for 10 minutes in a clinical centrifuge. A 2.4 ml aliquot of the organic layer was transferred to a new test tube and evaporated under argon gas. The residue was reconstituted in 100 µl methanol. An aliquot was analyzed on the HPLC system at λ = 240 nm and a Packard Flow Scintillation Analyzer (Meriden, CT) with a Waters Nova-Pak C18 column (3.9 x 150 mm, 4 µm particle size) with a Waters C18 Novapak guard column using the following solvent program where A = 35% methanol/64% H_2O/1% acetonitrile and B = 80% methanol/18% H_2O/2% acetonitrile:

Time	Flow rate (ml/min)	A	B
0	0.8	100%	0%
10	0.8	100%	0%
18	0.8	55%	45%
28	0.8	55%	45%
29	0.8	0%	100%
30	0.8	0%	100%
32	0.8	100%	0%
41	0.8	100%	0%

Testosterone, 6β- and 16β-hydroxytestosterone were identified by comparison with co-eluting standards and were quantified using the radioactivity of the peaks. Incubations containing no NADPH were run as a negative control.

Western blot analysis

Western blotting was performed based on a modified protocol (Ausubel et al. 2002). Ethanol and KimWipes were used to clean two 16x16 cm glass plates and were assembled with 0.75 mm spacers on a BioRad casting stand. The 8% separating gel solution contained 15.0 ml 1.5M Tris (pH 8.8), 150 µl 20% SDS, 6.35 ml milliQ H_2O, 8.0 ml 30% acrylamide (29:1), 0.6 ml 5% ammonium persulfate and 30 µl TEMED. It was applied with a sterile, disposable pipet along the edge of one of the spacers to 1 cm below the comb. The solution was topped with isopropyl alcohol to prevent exposure to oxygen which inhibits polymerization. The separating gel solution was allowed to polymerize at room temperature for 1 hour.

The isopropyl alcohol solution was poured off the polymerized gel and the space above the gel was dried with KimWipes. The 3% stacking gel solution contained 5.0 ml 0.5 M Tris (pH 6.8), 50 µl 20% SDS, 3.75 ml milliQ H_2O, 1.0 ml 30% acrylamide (29:1), 200 µl 5% ammonium persulfate and 10 µl TEMED. It was applied to the side of one of the spacers until the solution came to the top. A 0.75 mm comb with 15 teeth was carefully pushed into the solution to avoid trapping air bubbles. The solution was allowed to polymerize for 1 hour at room temperature. The comb was removed and excess gel was cut from

the top of the glass plates. The wells were washed three times with running buffer (final concentration of 25 mM Tris (pH 8.3), 192 mM glycine and 0.1% SDS). The assembled gel was removed from the stacking apparatus and attached to the upper buffer chamber of a BioRad PROTEAN II apparatus. Running buffer was added to the lower buffer chamber so that it covered 1 cm of the bottom of the glass plates and to the upper buffer chamber to cover the wells.

Samples and standards were boiled for 5 minutes at 95°C in sample loading buffer (1.2 ml milliQ H_2O, 0.2 ml 20% SDS, 0.25 ml 0.5 M Tris (pH 6.8), 0.2 ml glycerol, 0.1 ml β-mercaptoethanol and 50 µl 0.1% pyronin Y). Microsomal protein (20 µg/lane) and appropriate standards were separated by polyacrylamide gel elctrophoresis at 20 mA until the dye front reached the bottom of the gel. Running buffer was discarded. One of the spacers was used to pry open the glass plates. Excess gel from below the dye front, sides and stacking gel was removed. A small corner was cut out in the upper left corner to maintain gel orientation. The gel was removed from the glass plate and soaked in Towbin's transfer buffer (final concentration of 25 mM Tris base, 192 mM glycine and 20% methanol) for 30 minutes.

Proteins were transferred to a nitrocellulose membrane according to a previously reported method (Towbin et al. 1992). Scotch Brite pads, two sheets of Whatman (Maidstone, England) 3 MM filter paper and nitrocellulose membrane were soaked in Towbin's transfer buffer. The gel solution was disposed of and the gel was assembled into a transfer cassette as follows: (cathode electrode) Scotch Brite pad → filter paper → gel → nitrocellulose membrane → filter paper → Scotch Brite pad (anode electrode). Air bubbles were removed before latching by rolling a test tube over the surface. The tank was filled with transfer buffer and the separated proteins were transferred to a nitrocellulose membrane at 4°C overnight using a Bio-Rad Transfer Blot apparatus at 30V.

The nitrocellulose membrane was removed from the transfer cassette and washed for 5 minutes in H_2O. Nonspecific sites were blocked by incubating the membrane for one hour at room temperature in 2% BSA in PBS (160 mM dibasic potassium phosphate, 40 mM monobasic potassium phosphate, 8% NaCl at pH 7.4). Blocking solution was discarded. Five µg/ml of anti-CYP3A4/5/7, 2.5 µg/ml of anti-CYP2B1/2, 50 µg/ml of anti-FMO1, 24.8 µg/ml of anti-FMO3 or 25 µg/ml of anti-FMO5 antibodies made up in 2% BSA in PBS were incubated with the membrane for one hour at room temperature. Primary antibody was saved for subsequent membranes. After four 5 minute washes with PBST (PBS + 0.05% Tween-20), the membrane was incubated with secondary antibody (HRP-conjugated antibody against species the primary antibody was raised in (goat, mouse or rabbit)) in secondary antibody buffer (0.01M Tris, 0.8% NaCl, 0.5% Carnation milk powder, 0.05% Tween 20) at a dilution of 1:10,000 for one hour at room temperature. The membrane was then washed three times for five minutes and once for 25 minutes in PBST.

Binding of the antibodies was detected using a commercially available ECL kit and Hyperfilm (Amersham). Film was exposed for 30 seconds-5 minutes using a film cassette and was processed on a Kodak Xomat film developer. Bands were analyzed densitometrically using available software (ImageJ). Standards were used to calculate a standard curve by linear regression for protein concentration of the probed enzyme. Samples were compared against this curve to calculate protein concentration.

Statistics

Linear regressions and two-tailed *t*-test comparisons were carried out using Excel (Microsoft Redmond, WA) and SYSTAT software (SPSS Inc. Chicago, IL).

Results

Hepatic metabolism of SN by liver microsomes

To compare senecionine metabolism between sheep and cattle liver microsome incubations were conducted at equivalent concentrations of microsomal protein. After incubation, the metabolism products were separated by reverse phase HPLC. Figure 2.2 shows a typical reverse phase HPLC profile of senecionine metabolized by sheep liver microsomes. The chromatographic peak at 7.5 minutes corresponds to NADPH and those at 14.0, 21.0 and 29.0 minutes correspond to DHP, senecionine N-oxide and senecionine, respectively. Retention times were established with known standards. The *in vitro* metabolism of senecionine and the formation of its metabolites in sheep and cattle liver microsomes are shown in Table 2.1.

When both species were compared, the mean levels of DHP produced did not differ significantly ($p > 0.05$). The amount of parent compound remaining (SN_A/SN_B), the mean concentration of *N*-oxide formed and the ratio of DHP/*N*-oxide differed between both species ($p < 0.05$). The ratio of DHP to *N*-oxide allows examination of DHP formation relative to *N*-oxide formation. This is important when comparing metabolite formation between groups since the relative activities of the bioactivation (DHP) and detoxification (*N*-oxide) pathways are important in determining a species' toxicity to PAs. Sheep liver microsomes metabolized 25% of the available senecionine while cattle liver microsomes metabolized only 12%. Sheep liver microsomes formed over four times the amount of *N*-oxide (1.10 nmol/min/mg protein) during the incubation than cattle liver microsomes did (0.26 nmol/min/mg protein). This inversely affected the ratio of DHP/*N*-oxide in that the ratio was four times lower in sheep (ratio = 0.37) than in cattle (ratio = 1.68). Thus, cattle form more DHP relative to *N*-oxide than sheep do.

When animals were compared between genders within a species, cattle

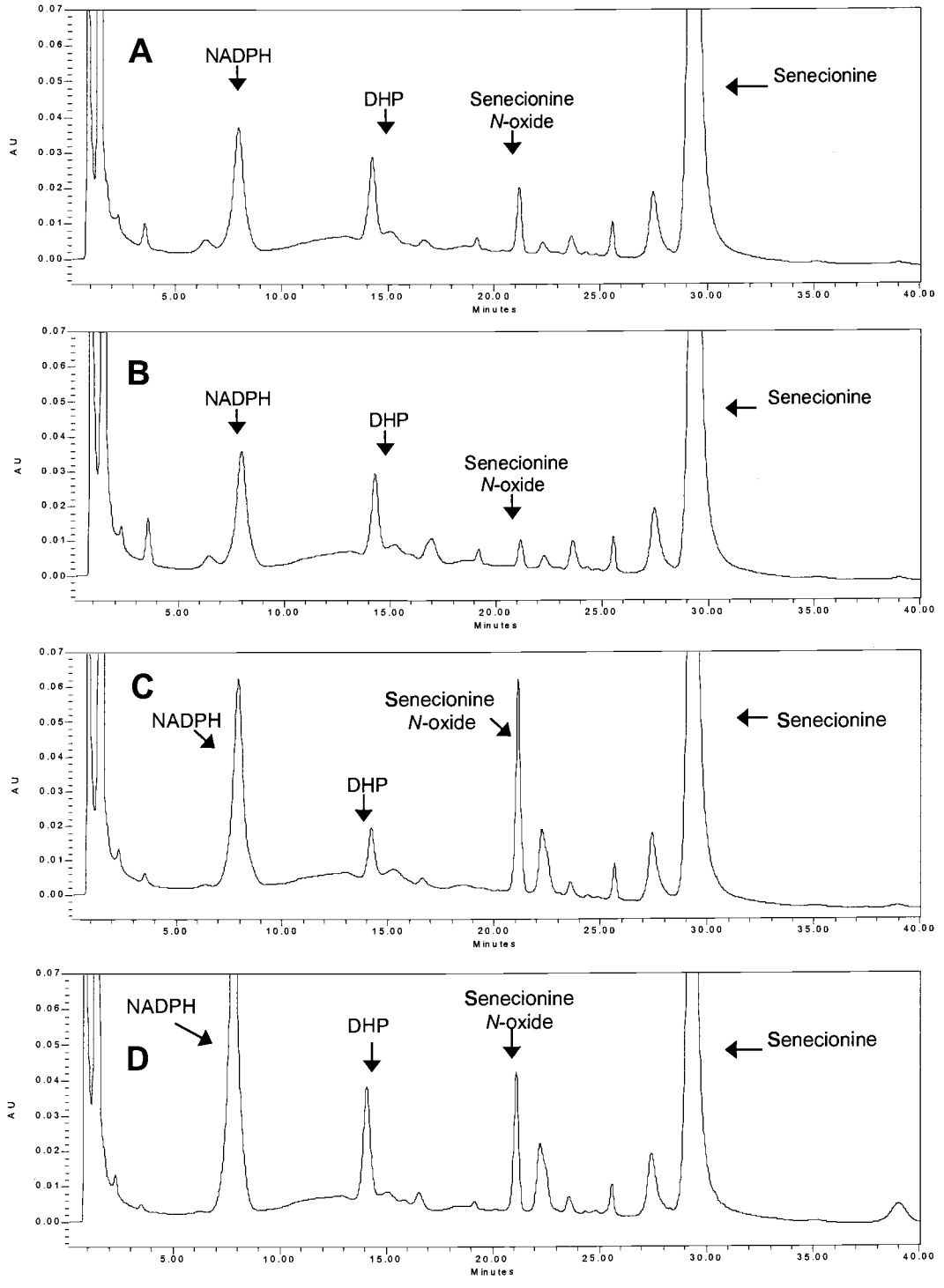

Figure 2.2 Representative reverse-phase HPLC analysis of liver of senecionine microsomal incubations by (A) female cattle, (B) male cattle, (C) female sheep, (D) male sheep liver microsomes.

Table 2.1 *In vitro* metabolism of senecionine by sheep and cattle liver microsomes to DHP and SN *N*-oxide.

Species*	Gender	DHP (nmol/min/mg protein)	SN *N*-oxide (nmol/min/mg protein)	Ratio of DHP/*N*-oxide	SN_A/SN_B† (%)
Cattle	Female	0.42±0.13	0.33±0.08	1.24±0.19	88.6±2.1
Cattle	Male	0.39±0.08	0.19±0.04	2.11±0.18	87.0±2.0
Cattle	*Both*	*0.41±0.01*	*0.26±0.07*	*1.68±0.29*	*87.8±1.9*
Sheep	Female	0.28±0.09	1.11±0.38	0.29±0.05	76.4±2.1
Sheep	Male	0.50±0.10	1.08±0.06	0.46±0.09	73.5±4.6
Sheep	*Both*	*0.39±0.11*	*1.10±0.26*	*0.37±0.08*	*74.9±3.4*

*Results are presented as mean ± SE of four animals from each gender run in triplicate according to the incubation conditions described. When both genders are combined, all eight animals are included in the data presented.
†SN_A and SN_B are expressed as amount of SN after (A) and before (B) metabolism.

liver microsomes showed a suggestive but inconclusive difference ($p = 0.06$) in the mean amount of *N*-oxide formed and a significant difference in the ratio of DHP to *N*-oxide. Female cattle produced more *N*-oxide (0.33 nmol/min/mg protein) than males (0.19 nmol/min/mg protein). This altered the DHP/*N*-oxide ratio so that females formed less DHP relative to *N*-oxide (ratio = 1.24), whereas males formed more DHP relative to *N*-oxide (ratio = 2.11). Sheep liver microsomes showed a significant difference between genders in the mean amount of DHP formed. Male sheep formed more DHP (0.50 nmol/min/mg protein) than female sheep (0.28 nmol/min/mg protein). This affected the DHP/*N*-oxide ratio so that male sheep formed more DHP relative to *N*-oxide (ratio = 0.46) than female sheep did (ratio = 0.29).

Linear regression was performed on the rate of formation of senecionine *N*-oxide versus DHP for all four groups and an r^2 of 0.86 was found for female cattle, 0.90 for male cattle, 0.83 for female sheep and 0.25 for male sheep (Figure 2.3). When an animal identified as an outlier was removed from the analysis for male sheep, the linear regression of senecionine *N*-oxide versus DHP had an r^2 of 0.82.

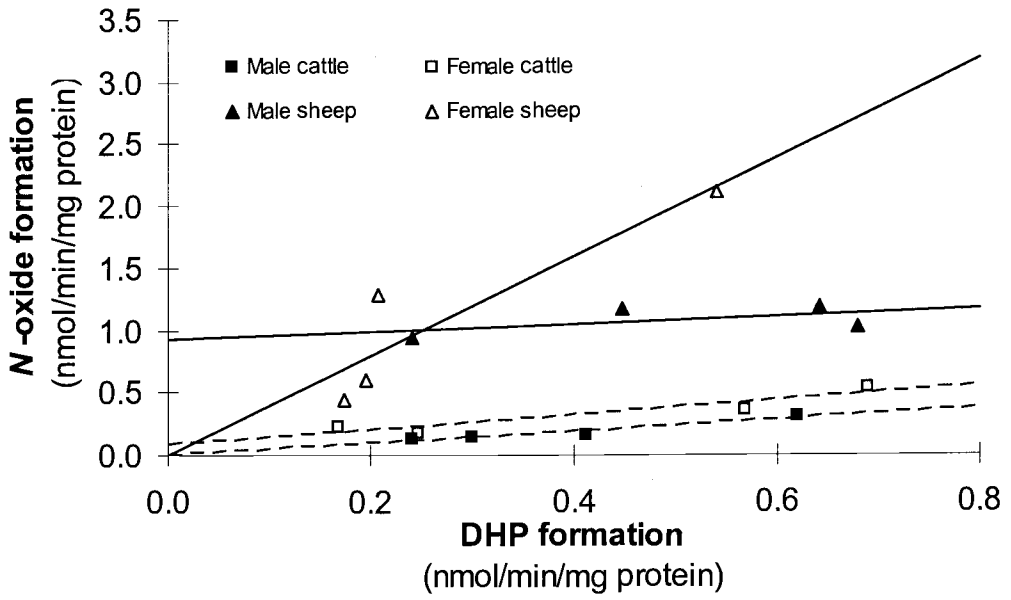

Figure 2.3 Correlation between rates of DHP and senecionine *N*-oxide formation from incubations with senecionine and sheep and cattle liver microsomes. r^2 was determined by linear regression.

Kinetic parameters of senecionine metabolism by liver microsomes

Enzymes are catalysts whose role is to increase the rate of a chemical reaction without being changed themselves (Mathews and van Holde 1996). Reactions can be defined by kinetics according to the Michaelis-Menten equation $V = V_{max}[S]/K_M + [S]$. V is the reaction rate or velocity, V_{max} is the maximum velocity of a reaction where all enzyme molecules are saturated with substrate, [S] is the substrate concentration and K_M is a ratio of the rate constants in a reaction. K_M is a measure of the substrate concentration required for effective catalysis to occur and is defined as the substrate concentration at which the reaction velocity has attained half its maximum value (1/2 of V_{max}). Thus, an enzyme with a high K_M requires a higher substrate concentration to achieve a given reaction velocity than an enzyme with a low K_M. K_M and V_{max} are characteristic of a reaction and can be measured through double reciprocal Lineweaver-Burke plots of 1/V versus

1/[S]. $1/V_{max}$ is the point of intercept with the 1/V axis. To find K_M, the linear relationship between 1/V and 1/[S] is extrapolated to the point where 1/V = 0.

Kinetic parameters (K_m, V_{max} and k_{cat}) of senecionine were determined for DHP and senecionine *N*-oxide formation. One representative microsomal preparation from each of the four groups (female cattle, male cattle, female sheep and male sheep) was run in triplicate in incubation experiments with senecionine concentration ranging from 0.05 to 1.0 mM. While one sample will not account for all interindividual differences in the metabolism of senecionine, the sample chosen for each of the four groups was closest to the mean formation of DHP and *N*-oxide values for each group. The results, including calculated K_m and V_{max} values, are depicted as Lineweaver-Burke plots in Figures 2.4A-H where 1/S = 1/[Senecionine] and 1/V = 1/reaction rate for DHP or senecionine *N*-oxide formation. Gender differences were most noticeable between male and female sheep. The V_{max} of *N*-oxide was 3.1-fold higher in female sheep than in male sheep, while the K_m of *N*-oxide was 1.8-fold higher. The K_m and V_{max} of DHP were 2.7- and 2.6-fold higher, respectively, in female than in male sheep. Cattle exhibited the largest gender difference for the V_{max} of *N*-oxide, with females exhibiting a value 1.7-fold higher than males. This suggests that female sheep have a larger metabolic capacity for senecionine than males, whereas cattle exhibit no difference between genders. When animals were grouped by species, the K_m and V_{max} values were strikingly different for *N*-oxide (Figures 2.5A-B). Sheep had a 23.8-fold higher V_{max} and 7.7-fold higher K_m than cattle for this metabolite.

Another way of expressing the catalytic efficiency of an enzyme is by examining its k_{cat} which is a direct measure of the catalytic production of product under optimum conditions (saturated enzyme). k_{cat} measures the number of substrate molecules turned over per enzyme molecule per second and is therefore termed the the 'turnover number.' It is expressed as V_{max}/K_m.

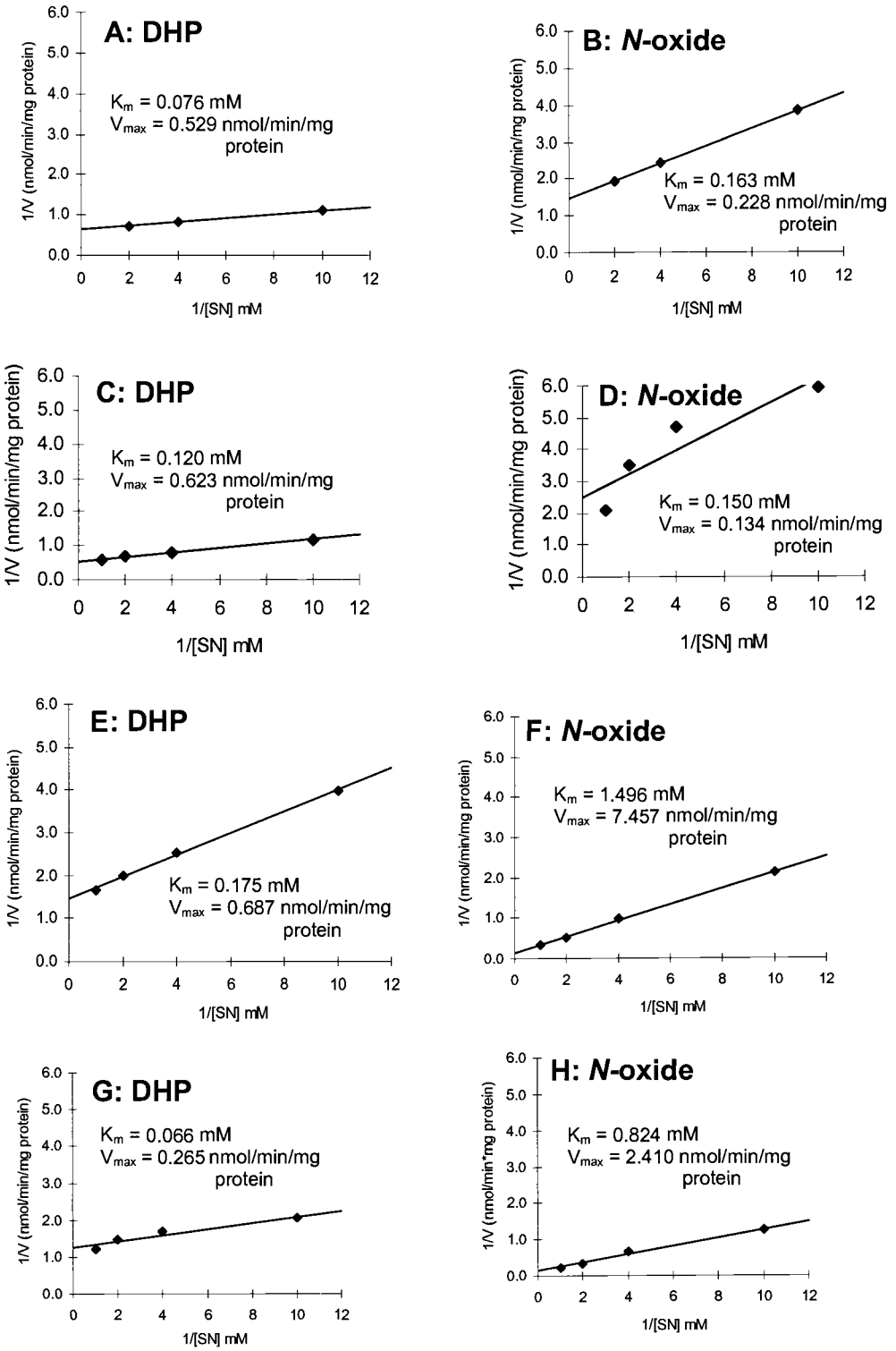

Figure 2.4A-H Kinetic analysis of DHP and senecionine *N*-oxide formation in female cattle (A,B), male cattle (C,D), female sheep (E,F) and male sheep (G,H) liver microsomes.

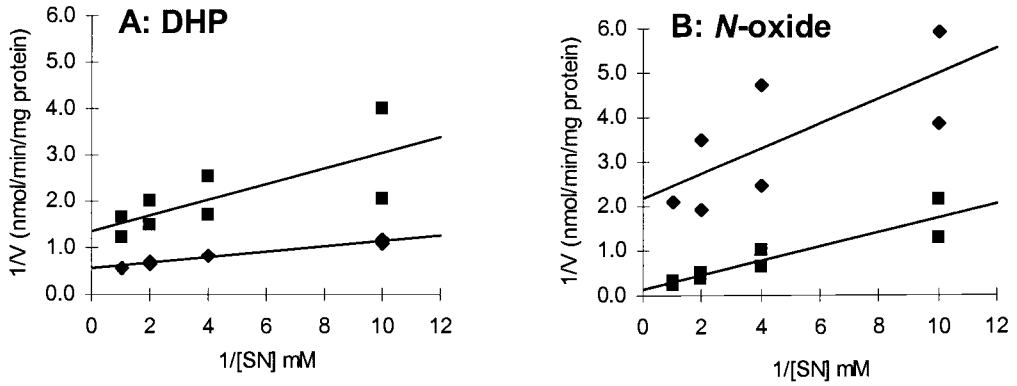

Figure 2.5A-B Kinetic analysis of DHP (A) and senecionine *N*-oxide (B) formation in sheep and cattle liver microsomes when all individuals are combined for analysis. Sheep = ■, cattle = ♦. (A) Cattle: K_m = 0.102 mM, V_{max} = 0.586 nmol/min/mg protein; sheep: K_m = 0.096 mM, V_{max} = 0.323 nmol/min/mg protein. (B) Cattle: K_m = 0.129 mM, V_{max} = 0.153 nmol/min/mg protein; sheep: K_m = 0.988 mM, V_{max} = 3.643 nmol/min/mg protein.

The k_{cat} for DHP formation was 6.96 s^{-1} for female cattle, 5.19 s^{-1} for male cattle, 3.93 s^{-1} for female sheep and 4.02 s^{-1} for male sheep. The k_{cat} for *N*-oxide formation was 1.40 s^{-1} for female cattle, 0.89 s^{-1} for male cattle, 4.98 s^{-1} for female sheep and 2.92 s^{-1} for male sheep. When animals are grouped by species, cattle had a k_{cat} of 5.75 s^{-1} for DHP formation while sheep had a value of 3.36 s^{-1}. *N*-oxide formation resulted in k_{cat} values of 1.19 s^{-1} for cattle and 3.69 s^{-1} for sheep.

Effect of chemical inhibitors on DHP and SN N-*oxide formation*

To determine the principal enzymes responsible for catalyzing the metabolism of senecionine to DHP and *N*-oxide, microsomal incubations were conducted in the presence of various inhibitors. The results of the chemical inhibition experiments are presented in Tables 2.2A-B. Table 2.2A contains results conducted under the incubation conditions stated above at the pH optimum for cytochrome P450s (pH 7.6), while Table 2.2B contains results from incubations performed at the pH optimum for FMOs (pH 8.5). At pH 7.6,

Table 2.2A Chemical inhibition of DHP and SN *N*-oxide formation from incubation with 0.5 mM senecionine and sheep and cattle liver microsomes at pH 7.6[†]

	Chemical Inhibitor											
	0.5 mM SKF-525A		10 µM Triacetyl-oleando-mycin		0.25 mM Methimazole		0.25 mM Thiourea		1.0 mM Phenylmethyl sulfonyl fluoride		0.1 mM Tri-o-cresyl phosphate	
	DHP	*NO*	*DHP*	*NO*	*DHP*	*NO*	*DHP*	*NO*	*DHP*	*NO*	*DHP*	*NO*
Species	*(as % control)*		*(as % control)*		*(as % control)*		*(as % control)*		*(as % control)*		*(as % control)*	
Female sheep	15.3± 3.1*	49.2± 10.6*	61.4 ±2.9*	67.4± 14.3*	37.6± 15.2*	21.3± 6.9*	40.6± 11.9*	39.0± 8.1*	87.3± 4.1	54.2± 13.7*	23.8± 2.9*	46.5± 9.8*
Male sheep	12.7± 4.1*	56.3± 3.3*	50.9 ±2.2*	83.6± 2.2*	15.1± 1.7*	9.7± 1.3*	24.5± 1.5*	41.4± 6.2*	90.2± 4.2	51.9± 7.3*	19.2± 2.9*	48.8± 3.6*
Female Cattle	22.1± 10.6*	48.7± 7.9*	17.6 ±0.7*	55.9± 11.3*	24.7± 11.0*	23.0± 7.1*	61.0± 7.2*	46.1± 6.2*	116.3 ±9.1	84.1± 19.1	25.1± 2.2*	47.6± 7.8*
Male cattle	12.6± 2.9*	35.9± 5.2*	18.0 ±2.7*	52.0± 9.7*	47.3± 13.0*	37.5± 6.2*	78.2± 10.6*	58.5± 6.4*	113.7 ±8.5	84.7± 10.9	21.4± 2.14*	51.3± 3.1*

[†]Values are expressed as % of control, mean ± SE of four animals and were performed in duplicate. In control, the average production of DHP and *N*-oxide was 0.28 and 1.11 nmol/min/mg protein for female sheep, 0.50 and 0.108 nmol/min/mg protein for male sheep, 0.42 and 0.33 nmol/min/mg protein for female cattle and 0.39 and 0.19 nmol/min/mg protein for male cattle.
*Designates inhibitor treatments that were significantly different from control ($p<0.05$)

Table 2.2B Chemical inhibition of DHP and SN *N*-oxide formation from incubation with 0.5 mM senecionine and sheep and cattle liver microsomes at pH 8.5[†]

	Chemical Inhibitor											
	0.5 mM SKF-525A		10 µM Triacetyl-oleandomycin		0.25 mM Methimazole		0.25 mM Thiourea		1.0 mM Phenylmethyl sulfonyl fluoride		0.1 mM Tri-*o*-cresyl phosphate	
	DHP	*NO*	*DHP*	*NO*	*DHP*	*NO*	*DHP*	*NO*	*DHP*	*NO*	*DHP*	*NO*
Species	*(as % control)*		*(as % control)*		*(as % control)*		*(as % control)*		*(as % control)*		*(as % control)*	
Female sheep	28.0± 4.0*	69.9± 3.5*	72.4± 3.4*	90.8± 1.3	35.3± 11.7*	16.4± 2.7*	35.4± 10.2*	26.0± 6.6*	98.0± 3.7	64.2± 7.2*	36.8± 6.1*	59.5± 9.2*
Male sheep	38.6± 9.8*	63.7± 2.0*	60.0± 4.1*	89.8± 3.0	25.3± 6.4*	4.8± 0.3*	25.4± 3.7*	19.6± 3.4*	110.1 ±9.1	65.4± 4.2*	41.5± 5.9*	59.5± 10.6*
Female Cattle	43.7± 8.5*	56.6± 1.9*	48.9± 11.5*	101.7 ±18.9	23.9± 8.3*	14.1± 4.4*	59.1± 15.8*	24.2± 5.3*	127.9 ±33.1*	95.3± 21.9	56.5± 12.2*	67.3± 14.0*
Male cattle	40.4± 4.5*	59.5± 4.3*	43.5± 6.5*	87.7± 8.5	39.1± 8.8*	22.8± 4.3*	71.6± 8.9*	34.4± 4.3*	123.8 ±16.2*	92.6± 5.4	42.0± 7.8*	52.4± 9.3*

[†]Values are expressed as % of control, mean ± SE of four animals and were performed in duplicate. In control, the average production of DHP and *N*-oxide was 0.28 and 1.11 nmol/min/mg protein for female sheep, 0.50 and 0.108 nmol/min/mg protein for male sheep, 0.42 and 0.33 nmol/min/mg protein for female cattle and 0.39 and 0.19 nmol/min/mg protein for male cattle.
*Designates inhibitor treatments that were significantly different from control ($p<0.05$)

the general cytochrome P450 inhibitor SKF-525A significantly inhibited DHP formation (13-22% of control) in both sheep and cattle and had less of an effect on *N*-oxide formation (36-56% of control). TAO, which specifically inhibits CYP3A (Huan et al. 1998b, Chang et al. 1994), had the largest effect on cattle who formed only 18% of the DHP formed by control incubations in both genders. Female sheep formed 61% of the DHP formed by control incubations while male sheep formed 51% when inhibited by TAO. The two FMO inhibitors (methimazole and thiourea) dramatically reduced *N*-oxide formation in both species at the pH optimum of 8.5. Methimazole reduced ormation most significantly in female sheep liver microsomes who formed only 16.4% *N*-oxide of control incubations, while male sheep, female cattle and male cattle formed 4.8%, 14.1% and 22.8% *N*-oxide of control incubations, respectively. Thiourea showed a respective formation of *N*-oxide of 26%, 20%, 24%, 34%. The carboxylesterase inhibitor PMSF showed little change in metabolism formation at pH 7.6. At pH 8.5, it increased DHP formation in cattle who showed a formation of 128% compared to control incubations in males and 124% in females. Female and male sheep formed 64% and 65% of the *N*-oxide formed by control incubations, respectively. The carboxylesterase inhibitor TOCP inhibited DHP formation at pH 7.6 in female sheep (24% of control), male sheep (19% of control), female cattle (25% of control) and male cattle (21% of control). It had less of an effect on *N*-oxide formation and at incubations conducted at pH 8.6.

Effect of immunochemical inhibitors on DHP and SN N-oxide formation

Antibodies raised against NADPH cytochrome P450 reductase, CYP3A, CYP2B, FMO1 and FMO3 were incubated with liver microsomes to further characterize the contribution of general (NADPH cytochrome P450 reductase) and specific (CYP3A, CYP2B, FMO1 and FMO3) enzymes in metabolism of senecionine to DHP and *N*-oxide. Due to the cost of inhibitory antibodies, only liver microsomes from the four representative metabolizers (as identified in the kinetic studies) were incubated with the antibodies and the maximum

concentration for NADPH cytochrome P450 reductase was 30 mg IgG/nmol P450 and 10 mg IgG/nmol P450 for the specific P450 and FMO isozymes. Results from Figure 2.6 show that there was a maximum inhibition in DHP formation by 46.1% in female cattle, 61.8% in male cattle, 69.0% in female sheep and 51.3% in male sheep liver microsomes. *N*-oxide formation was increased by 56% in female cattle, 32% in female sheep and 40.2% in male sheep liver microsomes while male cattle liver microsomes exhibited a 38% inhibition. Incubations were also performed with anti-CYP3A, -CYP2B, -FMO1 and -FMO3 antibodies. Due to the low concentration of antibody used (maximum concentration was 10 mg IgG/nmol P450), the general inability for anti-FMO antibodies to inhibit FMO enzymes and possible differences in interspecies specificity to the antibodies, however, the only significant inhibition for a specific monooxygenase enzyme was exhibited by antibodies raised against FMO3. It had a maximum inhibition of *N*-oxide formation of 20.5% for female cattle, 14.2% for male cattle, 24% for female sheep and 48.4% for male sheep liver microsomes (Figure 2.7). Interestingly, FMO1 showed a large increase in DHP formation for all four groups (Figure 2.8).

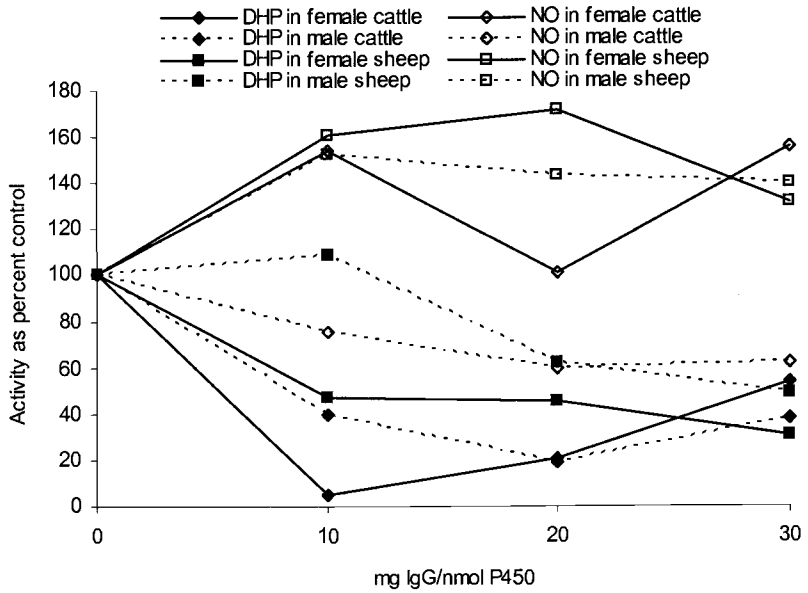

Figure 2.6 Immunoinhibition of senecionine metabolism to DHP and *N*-oxide by anti-rat NADPH cytochrome P450 reductase IgG.

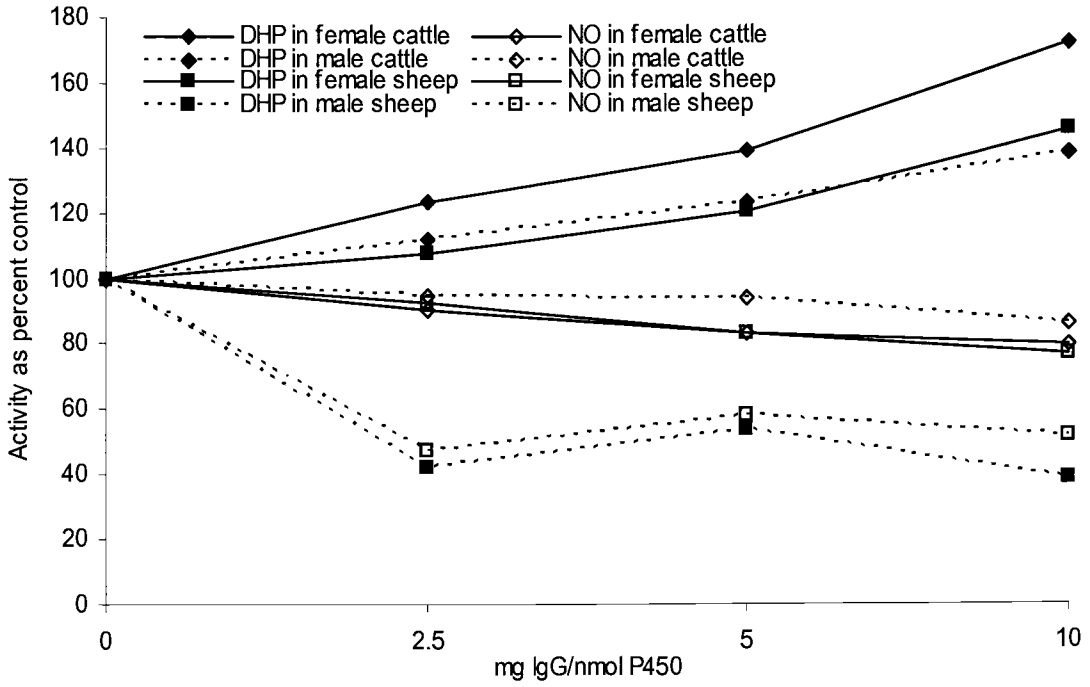

Figure 2.7 Immunoinhibition of senecionine metabolism to DHP and SN N-oxide by anti-human FMO3 IgG.

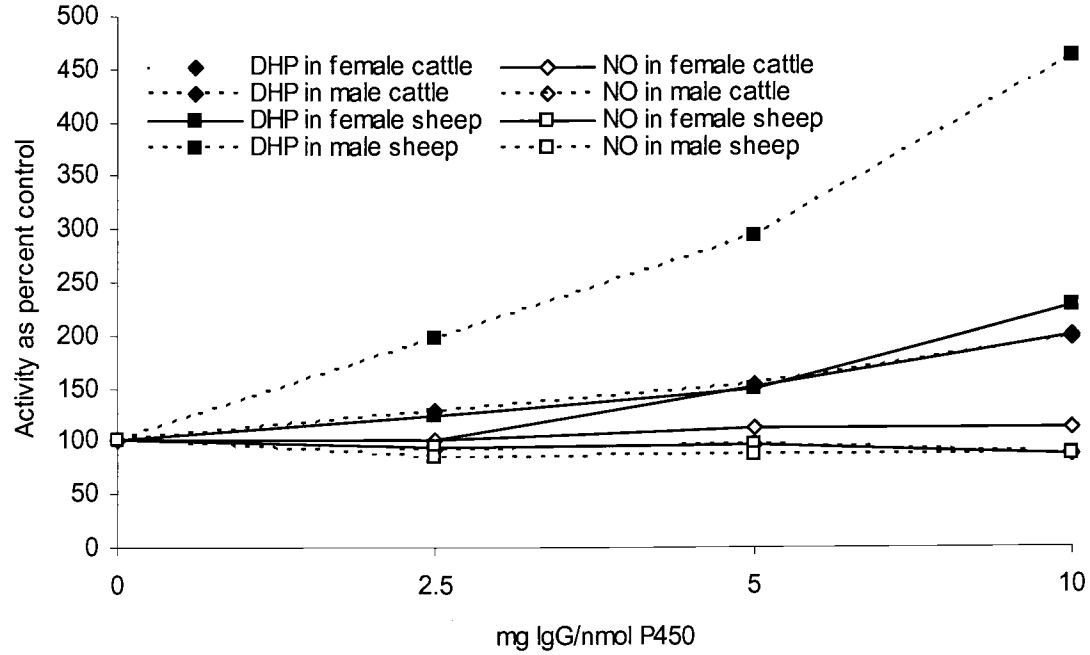

Figure 2.8 Immunoinhibition of senecionine metabolism to DHP and N-oxide by anti-human FMO1 IgG.

Female and male cattle produced 199% and 198% of the DHP in control incubations, while female and male sheep produced 228% and 461% of the DHP formed in control incubations at the maximum antibody concentration of 10 µg/nmol P450. *N*-oxide formation was 87%, 92%, 88% and 86% of control incubations for female and male cattle and female and male sheep, respectively. The results from incubations with anti-rat CYP2B and anti-mouse CYP3A are shown in Figures 2.9 and 2.10.

Methimazole-enhanced TNB oxidation

FMO catalytic activity was demonstrated by measuring the methimazole-enhanced oxidation of TNB (Figure 2.11). Sheep and cattle exhibited a significant difference in activity ($p<0.05$). Mean values were 3.1 nmol methimazole-enhanced TNB-oxidation/min/mg protein for cattle and 7.3 nmol methimazole-enhanced TNB-oxidation/min/mg protein for sheep. Gender comparisons within a species showed no significant differences: mean values were 3.5 for female cattle, 3.0 for male cattle, 7.2 for female sheep and 7.2

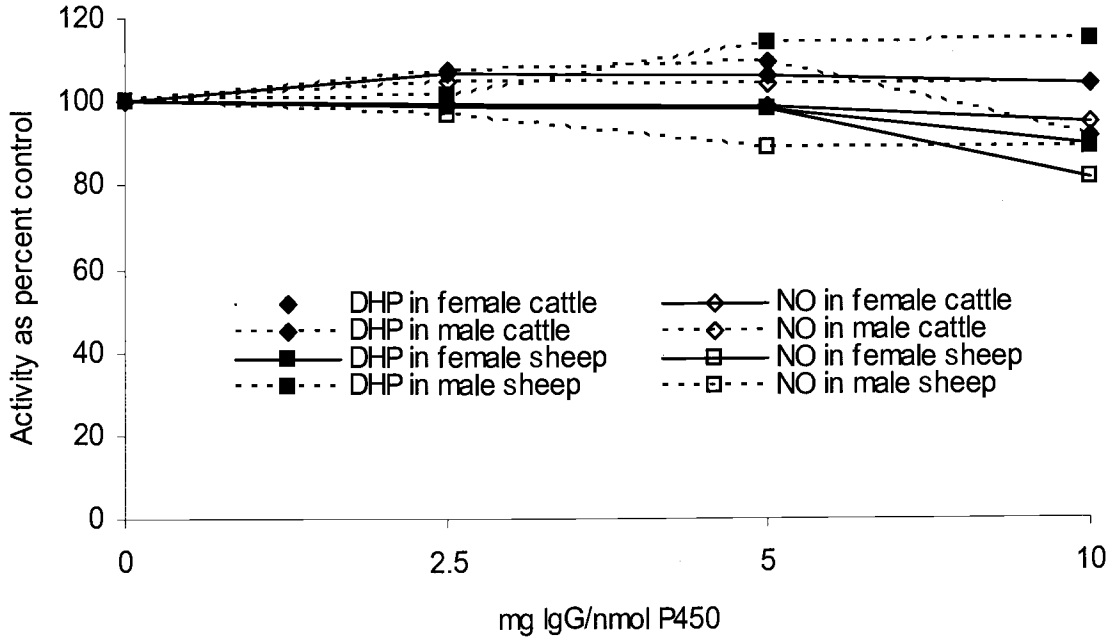

Figure 2.9 Immunoinhibition of senecionine metabolism to DHP and N-oxide by anti-rat CYP2B.

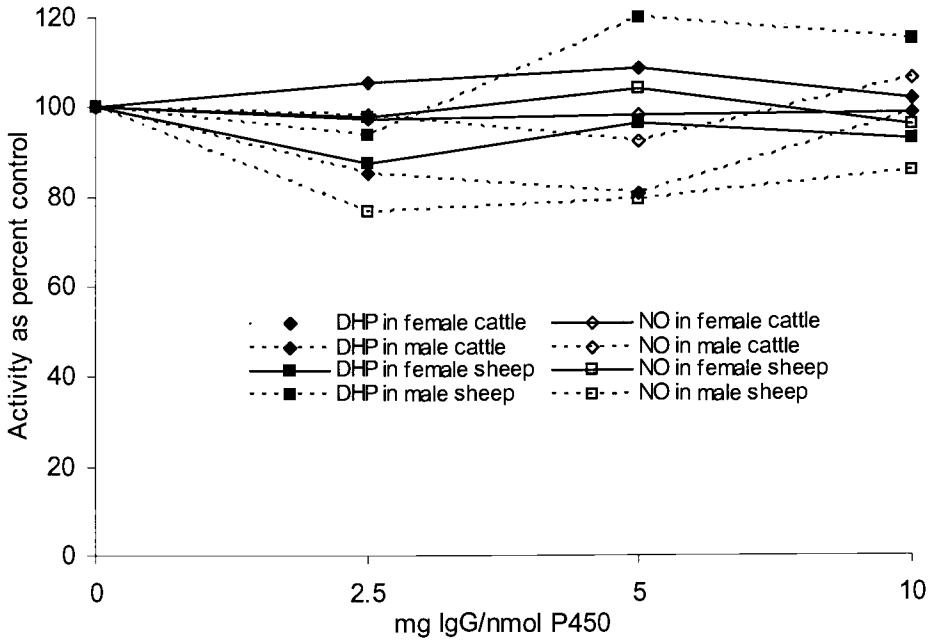

Figure 2.10 Immunoinhibition of senecionine metabolism to DHP and N-oxide by anti-human CYP3A.

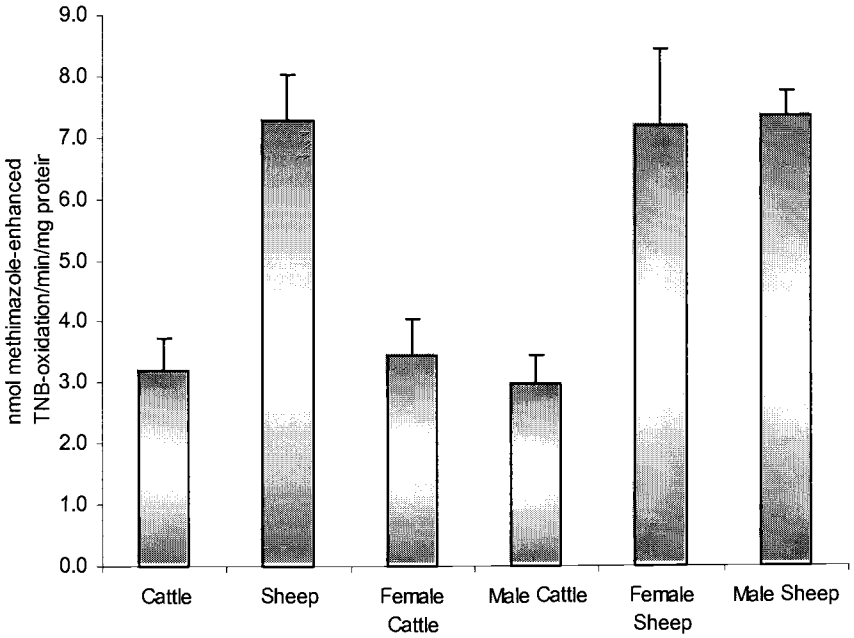

Figure 2.11 Methimazole-enhanced oxidation of TNB. Mean ± SE are represented as error bars. Analysis of eight animals was performed for the cattle and sheep. Analysis of four animals was performed for female cattle, male cattle, female sheep and male sheep.

nmol methimazole-enhanced TNB-oxidation/min/mg protein for male sheep.

Testosterone hydroxylation assay

The 6β- and 16β-hydroxylation of testosterone is reflective of the activity of CYP3A and CYP2B enzymes, respectively. When both products were measured in sheep and cattle, no significant difference was found either between species or between genders ($p<0.05$) (Figure 2.12). Cattle formed 0.160 nmol 6β-hydroxytestosterone/min/nmol P450 while sheep formed 0.214 nmol/min/nmol P450. Cattle formed 0.12 nmol 16β-hydroxytestosterone/min/nmol P450 and sheep formed 0.07 nmol/min/nmol P450. When separated into gender, female cattle formed 0.15 nmol/min/nmol P450 of 6β-hydroxytestosterone while male cattle, female sheep and male sheep formed 0.17, 0.20 and 0.23 nmo/min/nmol P450, respectively. 16β-hydroxytestosterone was formed at a rate of 0.09, 0.11, 0.08 and 0.06 nmol/min/nmol P450 for female cattle, male cattle, female sheep and male

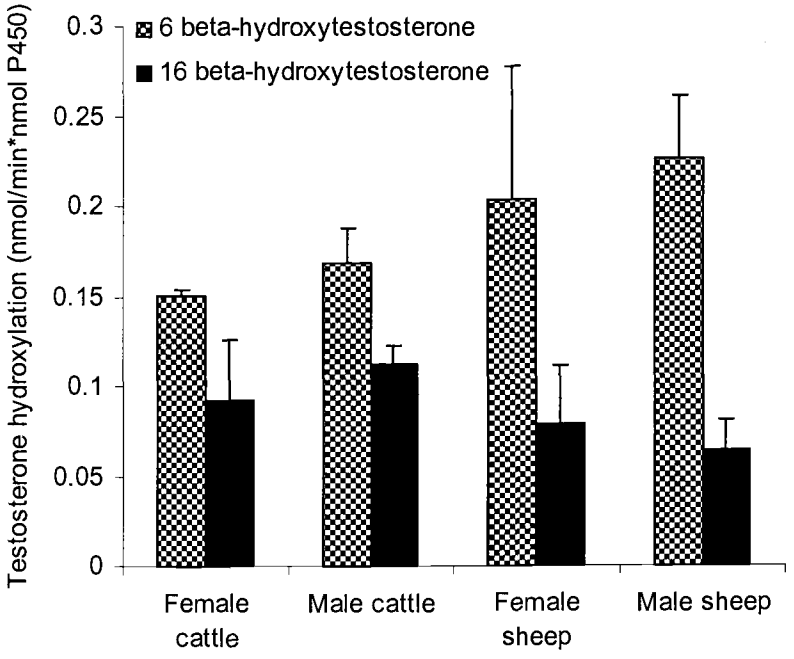

Figure 2.12 Testosterone metabolism by sheep and cattle liver microsomes. Incubations included four animals in each group and means ± SE are represented as error bars.

sheep, respectively. All four groups formed more 6β-hydroxytestosterone than 16β-hydroxytestosterone, with sheep showing the largest difference in activity.

Role of GSH in the metabolism of SN

Addition of GSH and/or cytosol is an indirect method of measuring the effect of GSH conjugation on SN metabolism. Addition of GSH alone resulted in a 79.5% reduction of DHP in female cattle, 73.0% in male cattle, 74% in female sheep and 79.4% in male sheep liver microsomes and had no significant effect on *N*-oxide formation (Figure 2.13). Addition of cytosol alone produced a 68.8% reduction in *N*-oxide formation in female cattle, 49.0% in male cattle, 62.9% in female sheep and 47.3% in male cattle liver microsomes and had less of an effect on DHP formation. Addition of both GSH and cytosol produced a reduction in both products. DHP formation was reduced by 83.6% in female cattle, 76.7% in male cattle, 78% and 81.5% in male sheep liver microsomes. *N*-oxide formation was reduced by 63.1% in female cattle,

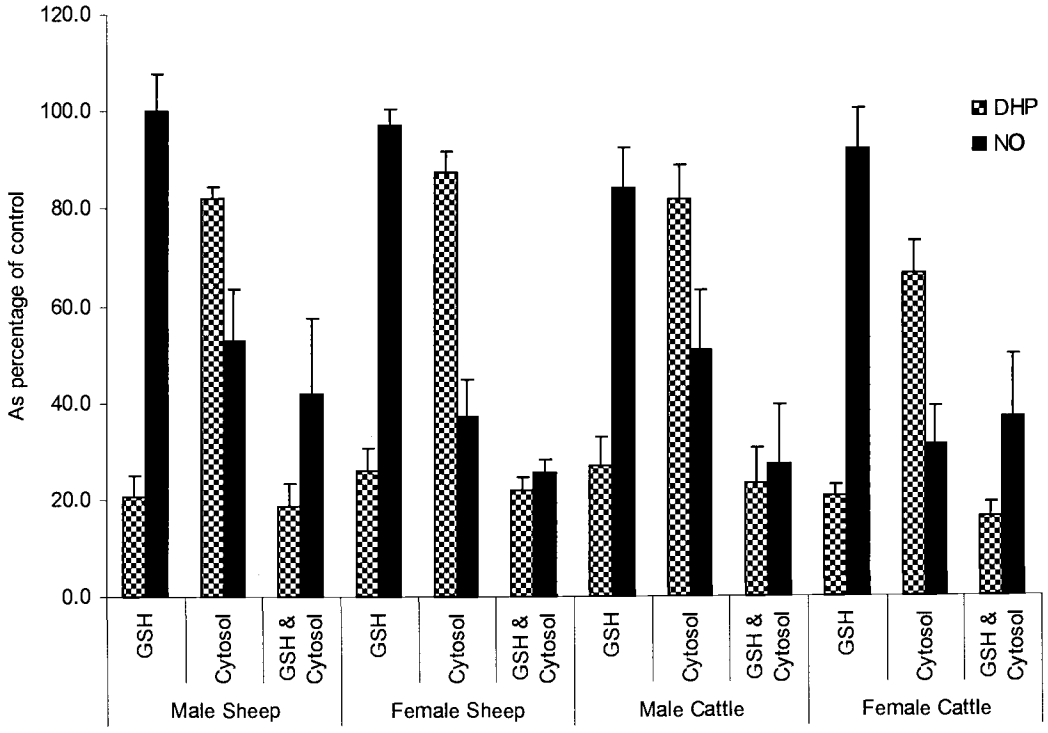

Figure 2.13 DHP and senecionine *N*-oxide formation from senecionine after incubation with glutathione, cytosol or glutathione and cytosol.

72.6% in male cattle, 74.4% in female sheep and 58% in male sheep liver microsomes.

When GST activity was measured, female cattle liver microsomes showed an activity of 38.5 nmol GST/min/mg protein for female cattle, 46.0 nmol GST/min/mg protein for male cattle, 83.0 nmol GST/min/mg protein for female sheep and 111.3 nmol GST/min/mg protein for male sheep. Values in liver cytosol were 285.5 nmol GST/min/mg protein for female cattle, 351.4 nmol GST/min/mg protein for male cattle, 1053.1 nmol GST/min/mg protein for female sheep and 1429.0 nmol GST/min/mg protein for male sheep. Thus, cattle GST liver cytosol concentration was approximately 7.5 times higher than GST liver microsome concentration. Sheep GST liver cytosol concentration was approximately 12.7 times higher than liver microsomes.

Western blot analysis

Western blots were performed on all 16 microsomal samples with each of the five antibodies (CYP3A4/5/7, CYP2B1/2, FMO1, FMO3 and FMO5). A molecular weight ladder (14.4-97.0 kDa) was run with each of the CYP3A4/5/7, CYP2B1/2, FMO1, FMO3 and FMO5 standards. The standards each showed a signal in the molecular weight range expected for P450 and FMO proteins (57-60 kDa). Measurement of CYP3A4/5/7 protein gave an average of 0.04 nmol/mg protein for cattle and 0.02 nmol/mg protein for sheep, which were significantly different from each other ($p<0.05$) (Figure 2.14). When genders were compared within species no differences were detected ($p > 0.05$). The primary antibody cross-reacted with proteins of a molecular weight around the 66 kDa molecular weight marker in some of the samples. This molecular weight is similar to that of NADPH cytochrome P450 reductase. After contacting the company that supplied the antibodies, it was confirmed that they may cross-react with NADPH cytochrome P450 reductase in some samples.

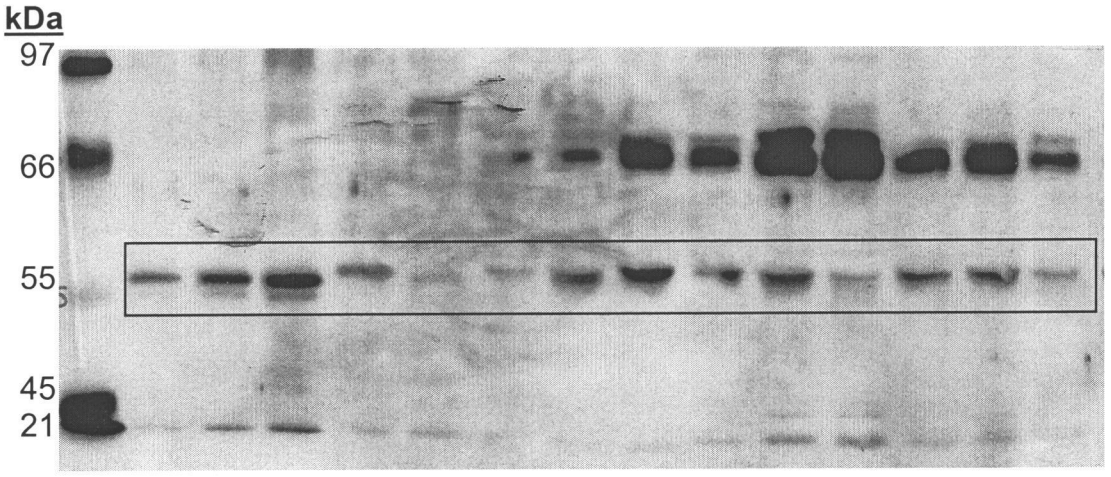

Figure 2.14 Western blot detecting CYP3A4/5/7 in sheep and cattle liver microsomes. The far left lane contains molecular weight markers. Lane identification: 1-3 = 0.5, 1, 2 pmol CYP3A4 standard; 4 = 1 pmol 3A5 standard; 5 = 1 pmol 3A7 standard; 6,10,11,14 = male sheep; 7 = female sheep; 8,13 = male cattle; 9,12 = female cattle. 20 µg of protein was loaded for each sample. The box identifies CYP3A4/5/7 proteins.

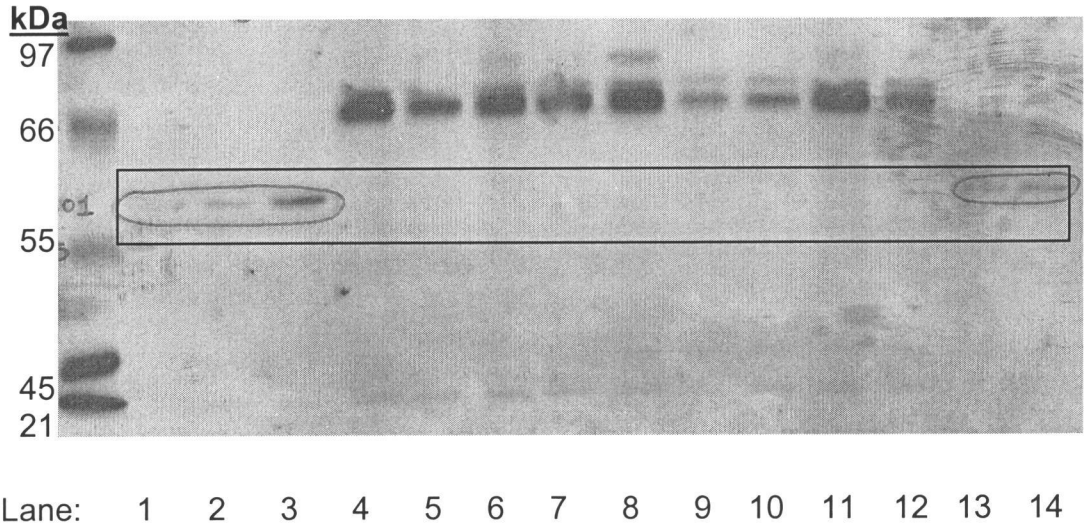

Figure 2.15 Western blot detecting FMO1 in sheep and cattle liver microsomes. The far left lane contains molecular weight markers. Lane identification: 1-3 = 0.02, 0.1, 0.2 µg FMO1 standard; 4,7,11 = male cattle; 5,6,8 = female cattle; 9 = female sheep; 10 = male sheep; 13-14 = 0.1, 0.2 µg FMO1 standard. 20 µg of protein was loaded for each sample.The box identifies FMO1 protein.

No signal was detected for the remaining antibodies for any of the 16 samples (example, Figure 2.15). Standards run for each isozyme were visible so the antibodies were responsive to their specific protein. Given the interspecies differences between the antibody (raised to rat and human antigens) and sample (ruminants), it is possible that the difference in sequence homology was too great to allow binding of the antibody to the separated proteins. Since no source of ruminant antibodies to these proteins was available at the time of these experiments, detection of these proteins will need to be investigated in the future when these antibodies become available.

Discussion

Livestock species susceptibility to plant toxins has been extensively studied on a variety of plant species. Pyrrolizidine alkaloids are a large and important family of natural toxicants produced by a variety of plant species. There is a marked variation in the susceptibility of animal species to the toxic effects of PA exposure. In ruminants, cattle are more susceptible whereas sheep are more resistant to PA toxicity. Susceptibility to PA toxicity is influenced by many factors including species, age and sex as well as other temporary factors such as the mode of PA administration, biochemical, physiological and nutritional status (Stegelmeier et al. 1999). The differences in toxicity in ruminants has been postulated to be due to transformation of PAs by microbes in the rumen or differential metabolism of PAs by hepatic enzymes. Digestion of PA contaminated forage in the rumen may transform the PAs to moieties that are less bioavailable for absorption and/or non-toxic. The number and detoxification ability of ruminal microbes at the time of plant ingestion, which has been discussed above, plays a significant role in an animal's ability to degrade the toxins present in PA-containing plants. Thus, the amount of total PA reaching the liver and detoxification of PA in the gut by microbial flora or gastrointestinal enzymes may provide an explanation for differences in toxicity

exhibited between species (Huan et al. 1998b). However, investigation of microbial degradation of PAs in sheep and cattle rumen fluid was beyond the scope of this study and has been investigated previously (Hovermale and Craig 2002, Craig 1995, Craig et al. 1992b, Craig et al. 1992a, Wachenheim et al. 1992a, Wachenheim et al. 1992b).

Once ingested by an animal, PAs can follow various mechanisms of disposal and their eventual biological effect depends on the balance of these pathways (Mattocks 1986c). Pyrroles (dehydrosenecionine and DHP) are highly reactive in alkylating cellular macromolecules and are described as the primary toxic metabolites in PA metabolism. Alternatively, PA *N*-oxides are not readily metabolized and are believed to be excreted unchanged in the urine within 24 hours due to their water solubility (Mattocks 1986c). Thus, *N*-oxide formation is said to be a detoxification pathway for elimination of PAs. Reaction with carboxylesterases results in the hydrolysis of PAs into necine and necic acid products, neither of which are hepatotoxic (Mattocks 1986c). Therefore hydrolysis is also known as a detoxification pathway.

This study investigated senecionine metabolism in sheep and cattle liver microsomes to characterize similarities or differences in hepatic biotransformation and detoxification pathways. Senecionine was chosen as the alkaloid to study *in vitro* hepatic metabolism in sheep and cattle since it is found in high abundance in tansy ragwort (*Senecio jacobaea*), a plant that is widely distributed in the rangelands of the Pacific Northwest. Senecionine is metabolized *in vitro* to DHP and senecionine *N*-oxide. There were quantitative differences in the extent of transformation and distribution of transformation products. This study confirmed previous findings by showing that the mean level of DHP formation did not differ between sheep (resistant) and cattle (susceptible) (Table 2.1), providing evidence that there is not a strong correlation between *in vitro* DHP formation from liver microsomes and a species' susceptibility to PA toxicity (Huan et al. 1998a). In addition, Huan et al. (1998a) found that the rate of senecionine disappearance was greater in

resistant animal species (sheep, hamster, rabbits) than in susceptible animal species (cattle, chicken, rat) as reported by Huan et al. (1998a). Sheep liver microsomes formed more *N*-oxide relative to DHP whereas cattle liver microsomes formed more DHP relative to *N*-oxide. Coupled with a higher rate senecionine disappearance (Table 2.1 and Figures 2.4 and 2.5), this could aid sheep in eliminating a potentially toxic compound through a relatively nontoxic pathway faster than cattle who form DHP at a much higher rate relative to *N*-oxide. When linear regression was performed on *N*-oxide versus DHP formation, most animals exhibited a correlation between the two, suggesting that both reactions could be catalyzed either by the same enzyme(s) or two closely related enzymes under the same regulatory control (Figure 2.3) (Miranda et al. 1991a).

When the K_m, V_{max} and k_{cat} of DHP and *N*-oxide formation were determined between species, the enzyme(s) responsible for *N*-oxide formation had larger values in sheep than in cattle (Figure 2.5). In sheep, these values were also larger for *N*-oxide than for DHP. Thus, the enzyme(s) responsible for the *N*-oxidation of senecionine has a greater capacity (higher V_{max}, K_m and k_{cat}) to utilize more of the parent compound for metabolism to *N*-oxide in sheep than in cattle. The enzymes responsible for DHP formation appear to have similar activity in both species.

Gender differences were also examined during senecionine metabolism. Cattle exhibited a suggestive difference between genders in *N*-oxide formation, as females produced almost twice the amount males did. This affected the DHP/*N*-oxide ratio such that males formed a higher amount of DHP relative to *N*-oxide than females did. In addition, male sheep formed more DHP than female sheep did. This affected the DHP/*N*-oxide ratio so that it was larger in male sheep than in female sheep. Thus, males formed more DHP relative to *N*-oxide than females did in the species studied. When the kinetic experiments were performed, sheep exhibited the most noticeable gender difference (Figures 4A-H). Females generally showed higher K_m, V_{max}

and k_{cat} values for both DHP and *N*-oxide formation, suggesting that the enzyme(s) responsible for DHP and *N*-oxide formation have a higher capacity for senecionine metabolism in female sheep. A previous study showed that rat liver microsomes exhibited a marked sex difference in *N*-oxidation of senecionine, owing to the presence of the male-specific CYP2C11 (Williams et al. 1989a). CYP2C11 is more efficient at senecionine *N*-oxidation than CYP3A1 which appears to carry out both the *N*-oxidation and formation of DHP. This suggested that female rats are more sensitive to PA toxicity than male rats due to their low rates of formation of *N*-oxide relative to DHP, which has been demonstrated previously (Mattocks 1986c). No clinical or histological differences in gender due to PA toxicity have been reported for cattle or sheep. Further investigation on gender differences in cattle and sheep using a larger number of samples needs to be conducted to determine if the apparent differences in *N*-oxide formation and kinetic measurements are due to gender-specific influences on monooxygenase presence or activity.

Chemical and antibody inhibitors can be used to identify the relative contribution of the enzymes they inhibit and have been used in many PA metabolism studies covering various species (Huan et al. 1998a, Huan et al. 1998b, Chung et al. 1995, Chung and Buhler 1994, Miranda et al. 1991b, Miranda et al. 1991a, Williams et al. 1989a, Williams et al. 1989b). SKF-525A, a general cytochrome P450 inhibitor, inhibited DHP formation so that only 13-22% of the DHP formed by control incubations was in seen in sheep and cattle (Table 2.2A). This suggests that P450s play a large role in the metabolism of senecionine to DHP in both species. Additional evidence for the important role of P450s is provided from the results of incubation with anti-NADPH cytochrome P450 reductase IgG (Figure 2.6), which inhibits all P450-catalyzed reactions. It showed inhibition of DHP formation amongst all four groups. TAO was used as a more specific P450 inhibitor to measure the role of the CYP3A family in DHP formation. TAO was found to have a large effect on cattle of both genders, showing only 18% of the DHP formation seen in

controls for that species. It had less of an effect on sheep, showing 61% of the formation seen in control incubations in females and 51% formation in males, making this the only inhibitor that exhibited a marked difference between species. Thus, CYP3A appears to play a larger role in DHP formation in cattle than in sheep. To date, there are no confirmed specific inhibitors of CYP2B in sheep (Huan et al. 1998b) and cattle. Therefore, the role of CYP2B enzymes in senecionine metabolism could not be fully evaluated through the use of selective inhibitors in this study.

Additional experiments were performed to further characterize the specific monooxygenases responsible for hepatic transformation of senecionine. Western blotting is used to specifically probe for and quantitate a protein of interest. Catalytic activity assays may miss enzyme-mediated activity if the animal had been previously exposed to dietary components that bind to the enzyme and inhibit its catalytic activity. Catalytic activity may also be too low and below the sensitivity of a given assay. Thus, methods such as Western blotting are employed to detect the nature and amount of protein. Since CYP3A is believed to be the main cytochrome P450 family responsible for PA metabolism to DHP, and in some cases to *N*-oxide (Huan et al. 1998a, Huan et al. 1998b, Chung and Buhler 1995, Miranda et al. 1992, Miranda et al. 1991a, Williams et al. 1989a), its presence and activity were further studied by Western blotting and catalytic activity assays. CYP2B has also been identified to play an important role in the bioactivation of senecionine in some species (Chung and Buhler 1995, Chung et al. 1995, Miranda et al. 1992), so the presence and activity of this isozyme was investigated as well.

An assay of the 6β- and 16β-hydroxylation of testosterone was used to measure the activity of CYP3A and CYP2B (Figure 2.12). The stereospecific site of testosterone oxidation has been attributed to P450 isozymes. Therefore, 6β and 16β-hydroxytestosterone were indirect measurements of CYP3A and CYP2B, respectively. No significant differences were found between species or genders, suggesting that activity of CYP3A and CYP2B

are similar in liver microsomes of both species (Figure 2.12). 6β-Hydroxytestosterone was formed at a faster rate than 16β-hydroxytestosterone in all groups, with sheep showing the largest difference in activity between the two metabolites. This suggests that CYP3A activity was greater than CYP2B activity in the metabolism of testosterone in sheep and cattle liver microsomes. However, western blot analysis of CYP3A showed a difference in enzyme concentration between species, with cattle liver microsomes containing over twice the amount of protein as sheep liver microsomes (Figure 2.14). This discrepancy between enzyme concentration and activity is an example of the effects other endogenous or exogenous compounds can have on the activity of monooxygenases. It is possible that other dietary components bound and inhibited CYP3A activity in cattle before they were euthanized. In effect, a proportion of the CYP3A enzyme in the microsomal prep was bound by other components which resulted in a decreased activity relative to the amount of enzyme present in the testosterone hydroxylation assay.

When activity of FMOs was measured by methimazole-enhanced TNB oxidation, sheep and cattle were found to differ significantly (Figure 2.11). Sheep exhibited over twice as much FMO activity than cattle did. Coupled with the observations from the *in vitro* metabolism and kinetic experiments for *N*-oxide above, this could partially explain the sheep's ability to form *N*-oxide at a higher rate than cattle since *N*-oxide formation is catalyzed mainly by FMO enzymes. Methimazole and thiourea were used to chemically inhibit FMOs at pH 8.5 and were found to drastically reduce *N*-oxide formation in both species and genders (Table 2.2B). This suggests that FMOs play a significant role in formation of senecionine *N*-oxide and therefore contribute to the detoxification of PAs in both species. This data is consistent with other studies (Huan et al. 1998a, Miranda et al. 1991b, Williams et al. 1989b). The inhibition in DHP formation by the FMO chemical inhibitors methimazole and thiourea could be explained be their ability to act as substrates for cytochrome

P450s which would occupy available P450 enzymes, resulting in lower metabolism of senecionine to DHP by these enzymes.

No significant detection of the anti-rabbit antibodies to FMO1, FMO3, FMO5 or the anti-rat CYP2B was found for the sheep and cattle liver samples assayed by Western blots in this study (example, Figure 2.15). However, based on information from previous studies on the presence of FMO (Huan et al. 1998a, Miranda et al. 1991b, Williams et al. 1989b) and CYP2B (Chung and Buhler 1995, Chung et al. 1995, Miranda et al. 1992) enzymes in other species and the presence of FMO and 16β-testosterone hydroxylation activity in this study, it is possible that the homology of the proteins between the species may not be conserved enough to enable specific binding of the non-ruminant antibodies used in this study to the separated sheep and cattle proteins. Due to the lack of availability of antibodies for P450 and FMO proteins in ruminant species at this time, detection of these proteins should be investigated at a later date when these antibodies can be purified.

In this study, the carboxylesterase inhibitor TOCP dramatically inhibited DHP formation (20% of controls) in all species and genders (Tables 2.2A and 2.2B). The other esterase inhibitor (PMSF) showed an increase in DHP formation in cattle only. An increase in DHP formation would be expected since more senecionine would be available for DHP formation if esterase activity was inhibited. Thus, neither TOCP nor PMSF appeared to be effective inhibitors of esterase activity in these experiments. Previous studies have shown that esterase hydrolysis may play an important role in PA metabolism (Huan et al. 1998a, Dueker et al. 1992a, Dueker et al. 1992b). One study purified two forms of hepatic carboxylesterase from guinea pigs to investigate the unusual toxicity of jacobine to this normally resistant species (Chung and Buhler 1995). They found that high pyrrole and low *N*-oxide formation from metabolism of jacobine, combined with low rates of hydrolysis of the purified esterases are the main reasons for jacobine toxicity to guinea pigs. Therefore, alternative methods need to be used to characterize the role of

carboxylesterases in metabolism of senecionine to retronecine and senecic acid in sheep and cattle.

This study supports the results of previous research which found that glutathione conjugation plays a significant role in the detoxification of PAs (Huan et al. 1998a, Yan and Huxtable 1995, Reed et al. 1992). Addition of 2.0 mM GSH resulted in a significant reduction of DHP formation, while cytosol had more of an effect on *N*-oxide formation (Figure 2.13). The combination of both components resulted in depression of both DHP and *N*-oxide formation. The decrease in DHP formation with additional GSH supports similar results found by Huan et al., 1998b which suggested that the relative amount of glutathione present in incubations can affect the production of DHP. Conjugation with the tripeptide glutathione to electrophilic xenobiotics is an important detoxification route utilized by many organisms for a wide array of toxins. The cellular concentration of glutathione is high (~10 mM), making nonenzymatic conjugation of xenobiotics a relatively significant pathway of elimination (Klaassen 1996).

An interesting finding in the GSH experiments was the decrease in *N*-oxide formation upon addition of cytosol (Figure 2.13). Glutathione-*S*-transferases catalyze the conjugation of some xenobiotics and can make up to 10% of total cellular protein. GST concentration differed dramatically between liver microsomes and cytosol from both cattle and sheep. Sheep cytosol contained 7.5 times more GST than sheep microsomes, while cattle cytosol had 12.7 times more GST than cattle microsomes. Liver cytosol contains many components that can play a role in detoxification including GSH, GSTs and carboxylesterases. It is possible that these components were present in the cytosol and were able to route senecionine through alternative metabolic pathways which decreased emphasis on the *N*-oxide detoxification pathway. Reed et al. (1992) showed that GSH reacts with the dehydrosenecionine pyrrole formed by dehydrogenation of P450s and not with the secondary DHP pyrrole. Thus, the additional GST could be conjugating dehydrosenecionine

thereby altering the amount of parent compound available for potential metabolism by the three defined metabolic pathways. Alternatively, DHP formation did not markedly decrease with the addition of cytosol so it is possible that a component present in the cytosol inhibited FMOs from forming *N*-oxide.

In summary, the ability to form more *N*-oxide relative to DHP, to metabolize more of the parent compound, to utilize esterases for conversion of PAs to nontoxic metabolites, to decrease the amount of toxic DHP formed when an adequate amount of glutathione is present, and to contain large quantities of PA-degrading microorganisms in the rumen are all mechanisms of resistance which sheep combine and use concurrently in order to abate PA toxicity. Cattle form more DHP relative to *N*-oxide than sheep, as exhibited by their high DHP to *N*-oxide ratio and low kinetic values for *N*-oxide formation, and metabolize the parent compound at a slower rate which exposes them to more toxic metabolites than sheep. The high abundance of the DHP-forming CYP3A enzyme present in cattle combined with their low levels of the *N*-oxide-forming FMOs may explain why they form more DHP relative to *N*-oxide. Hence, they may be exposed to more toxic metabolites than sheep as a result of differences in hepatic metabolism of PAs. In addition, cattle have the same species of PA-detoxifying microbes in their rumen but in much lower numbers than in the rumen of sheep (Wachenheim et al. 1992b). This increases the potential for PAs to be passed unmetabolized from the rumen to the liver.

Inhibition of cytochrome P450, flavin-containing monooxygenase and carboxylesterase activity was similar in sheep and cattle liver microsomes in the chemical inhibitor experiments. Further, both species were able to utilize the presence of glutathione in conjugation of DHP in a similar manner. Research has indicated that there is a relationship between diet, bacterial status and hepatic cytochrome P450 content in rats (Nugon-Bandon et al. 1998). This suggests that differential rumen microbial compositions of sheep and cattle are linked to the capabilities of the liver in both species to activate or

detoxify PAs. Combined with the findings of this study, it appears that species susceptibility is a complex interaction involving animal- and possibly gender-specific differences in both pre- and post-absorption metabolism (Stegelmeier et al. 1999).

A concurrent *in vivo* study involving sheep and cattle that looks at the effects of PAs on the microbial population in the rumen and their metabolism of PAs as well as liver samples profiling the presence and activity of Phase I and Phase II enzymes and their metabolism of PAs would allow a side-by-side comparison of both theories of susceptibility to PA toxicity. In addition, investigation of the gastrointestinal metabolism of PAs by Phase I and Phase II enzymes in the small intestine in sheep and cattle would provide more information on metabolism of PAs before they encounter monooxygenases of the liver. It has been suggested that *N*-oxides may not be a nontoxic endpoint compound and may in fact be converted back to the parent compound, with the possibility of entering one of the three metabolic pathways again (Couet et al. 1996). Therefore, an *in vitro* study using direct incubation of the *N*-oxide form of PAs would be of interest as many plants contain the bulk of PAs in this form and so would be important in terms of exposure to grazing animals.

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Chapter 3

SEMIQUANTITATIVE DETERMINATION OF ERGOT ALKALOIDS IN SEED, STRAW, AND DIGESTA SAMPLES USING A COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY

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Abstract

Ergot alkaloids present in endophyte-infected (E+) tall fescue cause fescue toxicosis and other toxic effects in livestock that consume infected plant tissue, leading to significant financial losses in livestock production each year. The predominant method currently in use for quantifying ergot alkaloid content in plant tissue is through high-performance liquid chromatography (HPLC), which quantifies the amount of ergovaline, one of many ergot alkaloids present in E+ plant tissue. The enzyme-linked immunosorbent assay (ELISA) method used in this study detects quantities of nonspecific ergot alkaloids and therefore accounts for greater amounts of the total ergot alkaloid content in E+ tissue than does HPLC. The ELISA can also be used to more expediently analyze a larger number of forage samples without sophisticated and costly analytical equipment and therefore could be more desirable in a diagnostic setting. The purpose of this study was to evaluate the between-day (daily) and within-run variability of the ELISA and to determine the binding efficiency of six ergot alkaloids to the 15F3.E5 antibody used in the competitive ELISA to ascertain its feasibility as a quick analysis tool for ergot alkaloids. A run is defined as one 96-well plate run under the described ELISA conditions. Straw samples had an average coefficient of variation (CV) for concentration of 10.2% within runs and 18.4% between runs, and the seed samples had an average CV for concentration of 13.3% within runs and 24.5% between runs. The grass tissue-based lysergic acid standard curve calculated from the ELISA had an average r^2 of 0.99, with a CV of 2.1%. Ergocryptine, ergocristine, ergocornine and ergotamine tartrate did not bind strongly to the 15F3.E5 antibody because of the presence of large side groups on these molecules, which block their binding to the antibody, whereas ergonovine and ergonovine maleate were bound much more efficiently because of their structural similarity to lysergic acid. Clarified rumen fluid was tested as an additional matrix for use in the ergot alkaloid competitive ELISA to determine whether livestock metabolism

experiments on the postingestion fate of ergot alkaloid in ruminants could utilize this assay as a quick screening tool for the presence of nonspecific ergot alkaloids in rumen fluid. HPLC and ELISA procedures were compared for their ability in determining ergot alkaloid toxicity based on the repeatability of the procedures and on the specific compounds they measure. The ratio of ELISA concentration to HPLC concentration (ergovaline) varied from 2.00 to 2.81 in seed samples and from 0.62 to 8.66 in straw samples, showing no consistent pattern between the two methods. Based on the lack of data at present for the identity of the toxin causing endophyte toxicosis and the lack of agreement between the ergovaline HPLC and ELISA analyses for ergot alkaloid, each method is equally valid as an indicator of toxicity and is the best means for determining the concentration of the specific toxin(s) they measure.

Introduction

Tall fescue (*Festuca arundinacea*) plants are often infected with the endophyte *Neotyphodium coenophialum* (Morgan-Jones and Gams), which is a fungus that confers benefits such as drought and stress tolerance and pest resistance to the plant while the plant provides energy and a sustainable environment for the fungus (Adcock et al. 1997). This beneficial symbiotic relationship between the plant and fungus is necessary to ensure optimal plant production and survival (Adcock et al. 1997). The presence of endophyte in the plant results in the production of ergot alkaloids, which are toxic metabolites of *N. coenophialum* and are represented by a group of compounds related to lysergic acid (Shelby and Kelley 1991). Ergot alkaloids cause a billion dollars in livestock damage each year in the United States alone (Oliver 1997) due to the induction of fescue toxicosis and other toxic effects exerted after livestock consumption of endophyte-infected plant material (Stuedemann et al. 1998, Craig et al. 1994, Shelby and Kelley 1991).

Clinical signs of fescue toxicosis include summer syndrome, fescue foot, and reproductive difficulties (Craig et al. 1994).

Compounds produced from endophyte-infected tall fescue include the ergopeptides ergovaline, ergovalinine, ergosine, ergonine, ergotamine, ergocristine, α-ergocryptine, β-ergocryptine, and ergocornine, the ergolines ergonovine, lysergol and lysergic acid amide, the pyrrolizidine alkaloids loline, *N*-acetyl loline, *N*-formyl loline, *N*-acetyl norloloine, and perlolidine, and the clavines chanoclavine, agroclavine, penniclavine, elymoclavine and 6,7-secoagroclavine. Ergot alkaloids from endophyte-infected tall fescue affect the vascular tissues and blood of grazing organisms by acting on α-adrenergic, serotonin, and dopamine receptors to cause a vasoconstrictive response (Oliver 1997, Oliver et al. 1993). As α-adrenergic agonists, ergot alkaloids may decrease respiratory rates in sheep and cattle. The ergopeptides also suppress prolactin secretion by binding to dopamine receptors; prolactin concentration is, therefore, often used as an indicator of fescue toxicosis in livestock (Thompson and Stuedemann 1993). Depressed serum prolactin in prepartum cattle can result in reduced milk yield, but has negligible effects once lactogenesis occurs. Conception and calving by heifers feeding on endophyte-infected hay is also decreased, leading to reproductive efficiency problems (Thompson and Stuedemann 1993).

In the spring of 1998, an export crisis occurred whereby straw exported from the United States to Japan was halted on request of the Japanese government because they believed US endophyte-infected straw to be toxic to their livestock. A resolution was formed that included obligatory testing of all exported straw for the endophyte toxins ergovaline (in tall fescue) and/or lolitrem B (in ryegrass). Because of this new requirement for the export of straw to Pacific Rim countries and the increasing number of straw growers who need certification to sell "nontoxic" straw to livestock producers, thousands of straw samples have been assayed for ergovaline or lolitrem B

concentration with high-performance liquid chromatography (HPLC) procedures.

Currently, the predominant method in diagnostic laboratories for determining ergovaline content in tall fescue plant tissue is HPLC (Craig et al. 1994, Rottinghaus et al. 1991). This method quantifies the amount of ergovaline, one of many ergot alkaloids in endophyte-infected tall fescue tissue (Yates and Powell 1988), in a given straw or seed sample. This HPLC method is both costly and time-consuming and requires the use of sophisticated analytical equipment and trained personnel. To more expediently analyze a larger number of forage samples for the quantity of total ergot alkaloids and to decrease analytical costs, a rapid and inexpensive assay would be useful for determining alkaloid levels in plants to assess whether they are safe for livestock consumption (Hill and Agee 1994).

Immunoassays are attractive for this task because of their sensitivity, rapidity, and use of relatively simple equipment (Hill and Agee 1994). An immunoassay for ergot alkaloids was developed to detect the presence of ergopeptine alkaloids when as little as 3% of the plants or 4% of the seeds are infected with endophyte (Shelby and Kelley 1991). The correlation coefficients between the HPLC and enzyme-linked immunosorbent assay (ELISA) data from this assay were lower than expected, which limited its use to qualitative assessments. A more sensitive quantitative assay was needed that could be used at low ergot alkaloid concentrations to better quantify alkaloids in plant tissue.

A specific monoclonal antibody (15F3.E5) for ergot alkaloids was developed and used in a competitive ELISA detection assay to screen for plants with low ergot alkaloid concentration (Adcock et al. 1997). Studies were conducted using this immunoassay to develop a more precise ergot alkaloid ELISA protocol, including specific determination of plant tissue extraction time, plant tissue mass, and extraction volume necessary for plant tissue samples that would produce repeatable ELISA results (Hill and Agee 1994). This assay

has been used to test for ergot alkaloids containing a lysergic ring moiety in plant tissue and urine (Stuedemann et al. 1998, Adcock et al. 1997, Hill and Agee 1994) and is optimal for determining endophyte toxicity due to ergoline alkaloids. No values have been reported for the variation seen specifically in ergot alkaloid concentration of seed or straw samples, however, nor have the between-run or within-run variations in the lysergic acid standard curve used in this assay been reported.

The purpose of this study was to evaluate these parameters and to determine the binding efficiency of six ergot alkaloids in comparison to lysergic acid. Clarified rumen fluid was also tested as an additional matrix for use in the ergot alkaloid competitive ELISA that utilizes the 15F3.E5 antibody. The ELISA measures a group of ergoline alkaloids with a lysergic acid moiety and thus accounts for a larger amount of total alkaloids, whereas the ergovaline HPLC only measures one ergopeptide. To determine which technique was, therefore, a better determinant of ergot alkaloid concentration and thus a better tool to negate the large loss in livestock production due to fescue toxicosis each year, the correlation between straw and seed ergot alkaloid concentration values obtained with the ELISA and the ergovaline concentration values obtained with HPLC was assessed.

Materials and Methods

Sample collection

Straw samples were collected from various grass growers in the Willamette Valley, Oregon. A core sampler was used to withdraw 20 cores randomly from different bales in each lot of straw immediately after harvesting, or straw was hand sampled in fields at 20 random locations. These core or hand samples were pooled for grinding once they were dried. Seed samples were obtained from seed standards used in HPLC analysis for ergovaline and were assayed by HPLC to determine ergovaline concentration.(Craig et al. 1994)

ELISA

Standard and Sample Preparation. The lysergic acid standards used to calibrate the standard curve for each plate were made fresh daily from a 1 mg/ml lysergic acid[a] in methanol stock solution by making a 25 ng/ml lysergic acid solution diluted with grass tissue-based EPBST phosphate buffer. EPBST buffer solution contained 1.17 g Na_2HPO_4, 8.175 g NaCl, and 500 µL polyoxyethylene-20-sorbitan monooleate in one liter of doubled-distilled water at pH 7.4. An endophyte-free straw sample (1.25 g) was added for every 100 ml of EPBST buffer and stirred for 5 min. The solution was incubated at room temperature for 15 min and then stirred again for 5 min. This solution was centrifuged at 1,520 x *g* for 10 min. The supernatant was removed and kept at approximately 2°C covered in the dark until used for making dilutions. The 25.00 ng/ml solution was then serially diluted with the grass tissue-based EPBST buffer solution to obtain four additional lysergic acid standards of 1.56, 3.13, 6.25, and 12.50 ng/ml each, done in triplicate. The lysergic acid standard curve (0, 1.56, 3.13, 6.25, 12.50, and 25.00 ng/ml) was run over 19 days.

The five ergot alkaloids ergonovine,[a] ergonovine maleate,[a] α-ergocryptine,[a] ergotamine tartrate,[a] and ergocristine,[b] were dissolved into methanol to obtain a 1 mg/ml stock solution. Ergocornine[b] was dissolved into 100% ethanol, because of its inability to go into solution with methanol, to obtain a 1 mg/ml stock solution. Each stock solution was used to make a 50 ng/ml solution of each ergot alkaloid diluted with grass tissue-based EPBST buffer solution. The 50 ng/ml solution was then serially diluted to concentrations of 0.025, 0.25, 2.5, 12.5 and 25 ng/ml for each alkaloid using the grass tissue-based EPBST buffer solution, in triplicate. Each alkaloid was run on three separate days at all five concentrations with a blank solution.

The extract from the 15 straw and six seed samples was made using an 80:1 (v/wt) solution (8 ml EPBST solution: 0.10 g ground plant material), which was vortexed and incubated for 30 minutes. The tubes were vortexed again

and centrifuged for five min at 1,520 x *g*. The supernatant was then removed, and straw or seed samples known from HPLC analysis to be high in ergovaline were further diluted 1:5 (supernatant:EPBST buffer solution). This solution was then dispensed directly into the microtiter wells in triplicate. Straw samples were run on two separate days in triplicate. Seed samples were run on a variable number of days in triplicate.

The clarified rumen fluid extract was produced by centrifuging strained ruminal liquor at 25,000 x *g* for 30 min and was stored at -13°C until used (Wachenheim et al. 1992). Rumen fluid lysergic acid standards were made by spiking clarified rumen fluid with the 1 mg/ml lysergic acid stock solution to obtain a 25.00 ng/ml lysergic acid in rumen fluid stock solution. The 1.56, 3.13, 6.25, and 12.50 ng/ml solutions were made by serial dilution of the 25 ng/ml solution with rumen fluid-based EPBST buffer solution. For the rumen fluid-based EPBST buffer solution, 1.25 ml of clarified rumen fluid was added for every 100 ml of EPBST buffer and stirred for 5 min. The solution was incubated for 15 min and then stirred for 5 min. The liquid solution was kept at 2°C covered in the dark until used for making dilutions. The rumen fluid lysergic acid standards (0, 1.56, 3.13, 6.25, 12.50, and 25.00 ng/ml) were run in triplicate over six days.

Protocol. The ergot alkaloid competitive ELISA protocol utilized monoclonal antibodies from the murine hybridoma cell line 15F3.E5 (Hill and Agee 1994). The following brief description is based on the protocol used in a similar study conducted with tall fescue plant tissue (Hill et al. 2000). A 96-well microtiter plate[c] was coated with 188 pg of human serum albumin conjugated with lysergol (Hill and Agee 1994) by incubating the microtiter wells overnight at 2°C with the protein conjugate dissolved in 50 µl of borate saline solution (6.19 g boric acid, 9.5 g $Na_2B_4O_7 \cdot H_2O$, 4.9 g NaCl at pH 8.5). The following day, the plate was washed three times with ELISA wash (1.21 g hydroxymethyl aminoethane, 500 µl polyoxyethylene (20) sorbitan monooleate, 0.2 g NaN_3 in 1 liter of double-distilled water, pH 8.0) on an automated plate washer.[d] The

wells were then blocked using 125 µl of bovine serum albumin blocking solution for 30 min on an orbital shaker[e] at room temperature.

The plate was washed three times with ELISA wash to remove excess blocking solution, and 50 µl of lysergic acid standard, rumen fluid standard, ergot alkaloid, straw sample, or seed sample was added to the appropriate wells in triplicate. Diluted (1:100) monoclonal antibody 15F3.E5 (50 µl) was subsequently added to the wells and allowed to incubate for 2 hours on the shaker, covered at room temperature. The plate was then washed three times with ELISA wash, and 50 µL of diluted (1:500) rabbit anti-mouse antibody-alkaline phosphatase[a] conjugate was added and allowed to incubate for 2 hr on the shaker, covered at room temperature. The plate was washed three times with ELISA wash, and 50 µl of *p*-nitrophenylphosphate[f] chromogen was added to each well and color permitted to develop for 20 min. Color development was stopped using 50 µl of 3 M NaOH,[g] and absorbance was measured at 405 nm[d]. The detection limit for the ELISA was 1.56 ng/ml for lysergic acid.

HPLC

Lysergic Acid. The samples were prepared by adding 5 ml of methanol to 0.25 g of the six seed and four straw samples. The tubes were allowed to rotate overnight in the dark. The following day, the samples were centrifuged at 1.52*g* for 10 minutes and 2.5 ml of the methanol supernatant was removed. This solution was dried under N_2 gas at 50°C. The samples were reconstituted in 0.5 ml of 0.001 N NaOH, filtered through a 0.22 µm filter, and run on the HPLC system. The controls were made by adding 20 µl of a 5.0 µg/ml lysergic acid in methanol stock solution to 0.25 g of an endophyte-free seed sample and prepared the same as the above samples. The 50, 100, and 200 ng/ml lysergic acid standards were made by diluting the 5.0 µg/ml stock solution. The 12.5 and 25 ng/ml lysergic acid standards were made by diluting

the 50 ng/ml standard solution. All solutionss were dried under N_2 gas at 50°C and reconstituted in 0.5 ml of 0.001 N NaOH and analyzed with HPLC.

Plant samples were prepared and analyzed for lysergic acid concentration by HPLC using a 250 series binary pump,[h] a 200 series autosampler[h] fitted with a 150-µl sample loop, an LC 240 fluorescence detector[h] with a 7-µl flow cell, an excitation wavelength of 250 nm and an emission wavelength of 420 nm, and a column packed with divinyl benzene (5 µm particle size). Data collection and quantification was from a PC-based system.[h] Mobile phase A consisted of 2.6 mM ammonium carbonate[i] and acetonitrile[g] (90:10 v/v), and mobile phase B contained 2.6 mM ammonium carbonate[i] and acetonitrile[h] (30:70 v/v) run at 1.0 ml/minute. The sample, control or standard (150 µl) was then injected into the system. After a five-min equilibration with mobile phase A, the program was linearly ramped to 100% B in five min, held at 100% B for 10 min, then linearly ramped down to 0% B in five min.

Ergovaline. Straw and seed samples were prepared and analyzed by HPLC following a previously described procedure (Craig et al. 1994). An isocratic mobile phase, an autosampler[h] fitted with a 20-µl sample loop, a fluorescence detector[h] with a 7-µl flow cell, and a column packed with divinyl benzene were used in the HPLC system for this procedure. Data collection was from a PC-based system[h]. All fifteen straw and six seed samples were analyzed by this protocol.

Statistical analysis

The percentage binding efficiency for each ergot alkaloid was calculated for each concentration value (0, 0.025, 0.25, 2.5, 12.5, 25 and 50 ng/ml for ergonovine, ergonovine maleate, α-ergocryptine, ergotamine tartrate, ergocristine, and ergocornine and 0, 1.56, 3.13, 6.25, 12.50 and 25.00 ng/ml for lysergic acid) by averaging the blank optical density (OD) value in a run and the OD value for a given concentration and then dividing the latter by the former to obtain the percentage binding efficiency (%BE). Each ergot alkaloid

was run on three separate days, and these three %BE values were averaged and plotted against molar concentration for all concentration values for each of the seven alkaloids. The 50% binding efficiency for all alkaloids was taken from this graph.

To obtain the within-run and between-run variation for straw samples, fifteen samples were run in triplicate on two separate days. A run is defined as one 96-well plate run under the described ELISA conditions. OD values and concentrations for each sample were obtained from the plate reader, which compares ODs of the straw samples to the OD versus lysergic acid concentration log-logit standard curve. From these data, the coefficient of variation (CV) for each sample was calculated separately for each run by dividing the SD of the concentration for the three replicates by the mean concentration of the three replicates. The within-run concentration was then obtained by calculating the average CV for all samples (separated into two runs). The between-run concentration was found by dividing the SD of the concentration for the six total replicates by the mean for the six replicates to get the CV for each sample. The average of these CV values was then taken to get the between-run variation for concentration. The same method was used to obtain the within- and between-run variation of the OD for straw samples, using OD in place of concentration. Seed sample variation was calculated in the same manner, except only six total samples were used in the calculations, and each sample was run a variable number of times.

The within-run and between-run variation of the grass tissue-based lysergic acid standard curve were determined by running six lysergic acid standards (0, 1.56, 3.13, 6.25, 12.50 and 25.00 ng/ml) nineteen times. Within-run variation was calculated by averaging the coefficient of variation for all six standards over 19 days. The between-run CV for each standard concentration was calculated by taking the SD of the standard concentration for all 57 replicates and dividing it by the mean concentration value for the 57 replicates to obtain the CV for that standard concentration. The six CV values were then

averaged to get the between-run concentration variation. The same method was used to calculate the OD within-run and between-run variation values. The average r^2 was found by averaging the r^2 values calculated by the plate reader from the lysergic acid log-logit standard curve for all 19 days. The rumen fluid-based lysergic acid standard curve within- and between-run variaiton and r^2 were calculated in the same manner over six days.

Statistical analyses of the data, including mean, SD, CV (SD/mean), %BE, and r^2 for standard curves, were conducted using commercially available software.[d,j,k]

Results

The fifteen straw samples analyzed by ELISA were also analyzed using the HPLC method for ergovaline. The resulting ratio of ELISA concentration (total ergot alkaloids) over HPLC (ergovaline) concentration varied from 0.62-8.66, with a CV of 54.7% (Table 3.1). (The six seed samples tested had a ratio range of 2.00-2.81, with an average ratio of 2.26 and a CV of 13.7%. The seed samples were analyzed a variable number of times by ELISA (Table 3.1). Lysergic acid and ergovaline were identified and quantitated from HPLC chromatograms of the four straw and six seed samples using the lysergic acid HPLC protocol. Lysergic acid eluted at approximately 8.5 minutes and ergovaline eluted at approximately 15.8 minutes and was extracted with an average extraction efficiency of 75% from this procedure. Quantitating these two peaks for lysergic acid and ergovaline concentrations was semiquantitative at best because of the poor repeatability among the samples in comparison to the ergovaline HPLC procedure, so these data are not reported. Other unidentified peaks were also eluted in the lysergic acid HPLC assay, but qualitatively this assay resulted in a much lower total ergot alkaloid concentration value (lysergic acid and ergovaline) than that obtained from the ELISA assay for these samples.

Four of the six ergot alkaloids tested did not exhibit strong binding using monoclonal antibodies from cell line 15F3.E5 in the competitive ELISA (Figure 3.1). Ergotamine tartrate, ergocryptine, ergocornine, and ergocristine did not bind well in this assay, as indicated by the high %BE across all concentrations for these compounds (Figure 3.1). Ergonovine and ergonovine maleate did show a decrease in binding efficiency with an increase in molar concentration; 50% binding efficiency occurs at approximately 10^{-7} mol/liter for ergonovine and ergonovine maleate, 10^{-8} mol/L for lysergic acid.

Fifteen straw samples were analyzed on two separate days, each run in triplicate by ELISA, with an average CV for concentration of 10.2% within

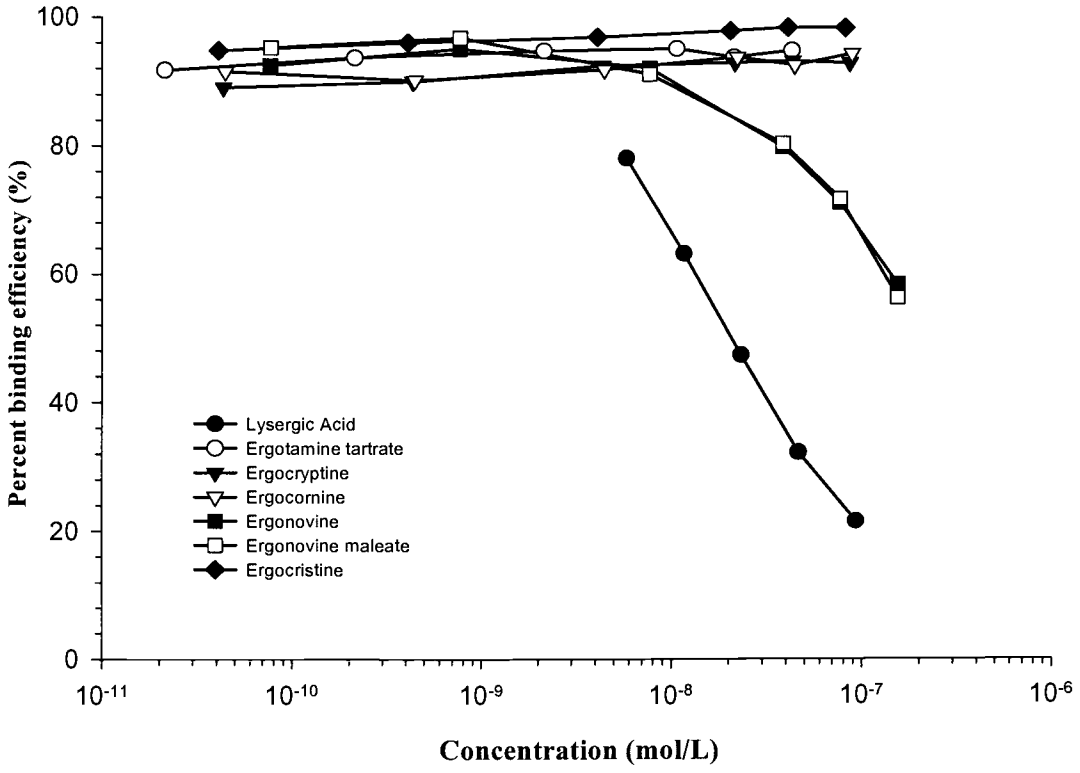

Figure 3.1 Comparison of the binding efficiency of ergot alkaloids run on a competitive ELISA assay using the 15 F3. E5 monoclonal antibody.

Table 3.1 Comparison of total ergot alkaloid ELISA concentration and ergovaline HPLC concentration values in 15 straw and six seed samples.

ELISA*			
Concentration (ppb)	***n***	**HPLC concentration (ppb)†**	**ELISA/HPLC**
Straw			
123 (120, 125)	...	200 (200)	0.62
1853 (1814, 1891)	...	214 (213, 214)	8.66
490 (478, 502)	...	254 (246, 261)	1.93
1990 (2384, 1596)	...	287 (287)	6.93
703 (678, 728)	...	306 (300, 311)	2.30
2377 (2965, 1789)	...	312 (311, 312)	7.62
915 (915, 915)	...	340 (338, 341)	2.69
2432 (3062, 1802)	...	375 (375)	6.49
681 (813, 548)	...	391 (386, 396)	1.74
1819 (2260, 1377)	...	403 (403)	4.51
2650 (3498, 1802)	...	425 (425)	6.24
2707 (2448, 2965)	...	459 (454, 463)	5.90
2181 (2371, 1990)	...	523 (513, 533)	4.17
2168 (2117, 2219)	...	708 (697, 718)	3.06
3291 (3425, 3156)	...	907 (885, 929)	3.63
Mean			4.43
SD			2.42
CV			54.7%
Seed			
35 (4-75)	5	0	...
219 (193-233)	3	76	2.81
390 (390)	1	162	2.41
1067 (831-1284)	4	522	2.04
2993 (2391-3888)	5	1500	2.00
6186 (5688-6563)	3	3000	2.06
Mean			2.26
SD			0.31
CV			13.7%

*Concentration as determined from the competitive ELISA (Hill and Agee 1994). The first number is the mean of the two replicates in parentheses for straw and the mean for the range of values in parentheses for seed (*n* is the number of times each seed sample was run by ELISA).

†Ergovaline concentration as determined by HPLC (Craig et al. 1994). The first number is the average of the two replicate values in parentheses for straw (some samples were not replicated). Seed samples are standards used in the ergovaline HPLC and were averaged over 15 runs.

Table 3.2 Within- and between-run average coefficient of variation for ergot alkaloid concentration and optical density from straw and seed forage samples run on competitive ELISA.

Sample type	Concentration			Optical Density		
	*n**	Within	Between	*n*	Within	Between
Straw	15	10.2%	18.4%	15	4.5%	5.9%
Seed	6	13.3%	24.5%	6	3.4%	17.5%

**n* = number of forage samples for a given sample type.

runs and 18.4% between runs (Table 3.2). The average CV of the OD for straw within runs was 4.5% and between runs was 5.9%. Six seed samples were run a variable number of times (see Table 3.1) and had an average CV for concentration of 13.3% within runs and 24.5% between runs. The six seed samples had an average CV for OD of 3.4% within runs and 17.5% between runs (Table 3.2).

Six lysergic acid standards of 0, 1.56, 3.13, 6.25, 12.50 and 25.00 ng/ml (equivalent to 0, 62.5, 125, 250, 500, 1,000 ppb before dilution) were used in calculating a log-logit standard curve for each run, which was then used to quantitate straw and seed samples in the same run by the plate reader. The grass tissue-based lysergic acid standard curve was run 19 times and had an average within run coefficient of variation of 36.1% and a between run average CV of 36.1% within runs and 49.1% between runs (Table 3.3). The average r^2 for the log-logit standard curve was 0.99, with a CV of 2.1%. The same six lysergic acid concentrations were diluted into a rumen fluid-based solution and used to calculate a rumen fluid standard curve. This standard curve was run six times and had an average CV of 34.8% within runs and a 39.8% between runs. The average r^2 was 0.98, with a CV of 5.0% (Table 3.3).

Table 3.3 Within- and between-run variation of the grass tissue-based and rumen fluid-based lysergic acid standard concentration curves run on competitive ELISA using six lysergic acid standards of 0, 1.5625, 3.125, 6.25, 12.5, and 25 ng/ml.

Standard Medium	*n**	CV†		Average r^2 (CV)‡
		Within Run	Between Runs	
Grass Tissue	19	36.1%	49.1%	0.99 (2.1%)
Rumen Fluid	6	34.8%	39.8%	0.98 (5.0%)

* *n* = number of standard curves calculated with six lysergic acid standards.
† CV = coefficient of variation.
‡ Average r^2 for all *n* runs. Number in parentheses indicates the CV for the average r^2.

Discussion

Technical difficulties encountered when measuring ergot alkaloids in *Neotyphodium coenophialum*-infected tall fescue are compounded by a general lack of understanding of metabolism and toxicities of the ergot alkaloids and their derivatives in livestock. Debate on whether HPLC or ELISA techniques are better for determining the toxic potential of ergot alkaloids is based upon issues related to repeatability of the procedure and toxicities of the compounds they measure. Ergovaline is commonly referred to as the toxic element within tall fescue because it is the predominant ergopeptine form of the ergot alkaloids found in *N. coenophialum*-infected tall fescue tissue (Yates and Powell 1988, Lyons et al. 1986). This observation is based upon an HPLC analytical procedure that does not analyze for the simpler ergoline alkaloids (i.e. lysergic acid, D-lysergic acid amide, lysergol, and ergonovine) or other unknown lysergic acid amides that may be present in *N. coenophialum*-infected tall fescue. Furthermore, HPLC analysis of ergopeptine alkaloids has

done little to elucidate their metabolic fates post ingestion. The metabolic half-life in serum is approximately 10 minutes for ergopeptine alkaloids and 40 minutes for a lysergic acid amide when measured by HPLC (Moubarak et al. 1996).

An advantage of the ELISA is that it detects greater quantities of nonspecific ergot alkaloids than does HPLC in seed and vegetative tissues, hence accounting for greater amounts of the total alkaloid content of *N. coenophialum*-infected tall fescue. Monoclonal antibodies from cell line 15F3.E5 bind ergot alkaloids based on a lysergic acid ring structure (Stuedemann et al. 1998). The ergopeptides ergocryptine, ergocristine, ergocornine, and ergotamine tartrate have the same main ring structure as lysergic acid but have additional large side groups that prevent them from successfully binding to the antibody, so that ergopeptides are not effectively measured by the ELISA. Ergonovine and ergonovine maleate exhibited a decrease in binding efficiency with an increase in concentration, which indicates that these two compounds reacted more strongly with the monoclonal antibody and had a chemical structure more similar to that of lysergic acid than to that of the other four ergot alkaloids. The binding efficiency of ergovaline was not determined because of the limited availability and high cost of its pure form; however, previous studies have shown that ergovaline exhibits a low binding affinity to the 15 F3.E5 antibody (molar concentration for 50% maximum absorbance value of 2.40×10^{-9} for ergovaline vs. 3.72×10^{-10} for lysergic acid and 3.93×10^{-15} for lysergol (Hill and Agee 1994), following the reasoning that large side groups on the ring structure prevent them from binding to the antibody.

Hybridoma cell lines with affinities to the ergoline alkaloids (15F3.E5) and ergopeptine alkaloids (9A12 and 9B1) have been used to examine the metabolic fate of the ergot alkaloids in animals grazing tall fescue (Stuedemann et al. 1998). Antibodies from the hybridoma (15F3.E5) accounted for approximately 95% of total excreted ergot alkaloids; urine was

the primary means by which the toxins were voided from the body. Urinary alkaloid concentration, as determined from antibodies of hybridoma 15F3.E5, were less variable than serum prolactin and may be a better physiological diagnostic tool than prolactin for fescue toxicosis in livestock (Hill et al. 2000). Urinary alkaloid excretion values, as determined by ELISA using antibodies from hybridoma 15F3.E5, were also highly correlated with average daily gains for steers grazing tall fescue (Hill et al. 2000). Therefore, an ELISA using antibodies from hybridoma 15F3.E5 may provide an estimate of an unknown ergot alkaloid component that is capable of eliciting a toxic effect in livestock grazing tall fescue.

In this study, there was a significant amount of day-to-day variance for the ELISA results (Tables 3.2 and 3.3), which has been noted in previous studies (Hill and Agee 1994), suggesting that the ELISA should be used as a semiquantitative tool. Within-run variation was lower because all sample extracts and standard concentrations were made new for each run and were exposed to the same experimental conditions on one microtiter plate. The competitive ergot alkaloid ELISA was adaptable to other matrices, such as rumen fluid, as indicated by the comparable amount of variation between the grass tissue-based standard curve and the rumen fluid-based standard curve (Table 3.3). However, the binding efficiency of different ergopeptine alkaloids is important to consider when screening rumen samples for the quantification of total ergot alkaloids in a diagnostic or research setting.

One explanation for the discrepancy in results between the HPLC and ELISA alkaloid determination in this study is the possibility that monoclonal antibodies from the hybridoma cell line cross-reacted with plant products other than the ergot alkaloids. However, cross-reaction is unlikely based upon direct evidence as to the affinity responses of the antibodies and direct and circumstantial evidence that the antibody has affinity to the toxins causing toxicosis. First, the monoclonal antobodies produced by murine hybridoma cell line 15F3.E5 were elicited after immunizing mice with a protein conjugate,

using lysergol as the hapten (Hill and Agee 1994). The antibodies were tested for affinity to lysergic acid amides and ergopeptine alkaloid derivatives. The antibody expressed some cross-reactivity to all ergot alkaloids with the exception of di-hydroergocornine or bromo-ergocryptine. Both of these alkaloids have substitutions at the 9,10 double bond of the lysergic acid ring structure, thus changing the three dimensional conformation of the lysergic ring slightly by changing the bond angle. This conformational change resulting from the hydrogenation or bromation of the 9,10 double bond resulted in zero affinity to the antibody by these alkaloids. Therefore, the antibody is highly specific to alkaloids with intact lysergic ring moieties only. Second, the endophyte-free grass and seed samples used in this study gave values at or near zero, a response that could happen only if the antibodies did not recognize plant products. Hence, there appears to be no evidence supporting cross-reaction to plant products that are not associated with the endophyte.

Some semidirect evidence exists to suggest that monoclonal antibodies from murine cell line 15F3.E5 have affinity to the toxin responsible for causing fescue toxicosis. First, when the antibody was infused into steers grazing endophyte-infected tall fescue, serum prolactin increased within 30 minutes after initiation of infusion of the antibody. Serum prolactin is the physiological marker most often used to diagnose fescue toxicosis, and it is highly probable that the antibody is recognizing the toxin(s) associated with this biochemical effect of the toxicosis syndrome (Hill and Agee 1994). Second, urinary excretion of ergot alkaloids quantitated using the same competitive ELISA as used in the present study was inversely correlated with steer average daily gain values (r^2=0.86) (Hill et al. 2000). Conversely, no similar data have been obtained supporting ergovaline as the toxin causing fescue toxicosis.

The poor affinity of 15F3.E5 to the ergopeptine alkaloids noted herein and by others (Hill et al. 2000), the lack of agreement between ergovaline HPLC and the ELISA for ergot alkaloids, the evidence supporting specificity of 15F3.E5 to the toxin(s) causing the anomaly, and the precision of the

ergovaline HPLC versus the ELISA in quantifying the alkaloids presents an interesting puzzle as to which test is the best measure of the potential of plant tissue to cause fescue toxicosis. Ergovaline HPLC is highly reproducible and is the best measure of this compound, but there is little or no evidence supporting ergovaline as the candidate toxin other than an association between it and endophyte-infected plant tissue and its vasoconstrictive effects. Moreover, it is not known whether ergopeptine alkaloids are modified by rumen bacteria nor which ergot alkaloid classes cross the rumen barrier; however, urinary concentrations of alkaloids as determined from ELISAs have been relatively high (Hill et al. 2000).

Conversely, ELISA of ergot alkaloids using monoclonal antibodies from murine cell line 15F3.E5 produces variable results and is thus semi-quantitative, but data exist suggesting affinity to the candidate toxin(s) causing the toxicosis. The ELISA and the ergovaline HPLC measure different alkaloid classes, but because the relationship of individual ergot alkaloids in causing endophyte toxicosis is unknown, each method is a valid indicator of toxicity and the best means for quantifying the specific compounds they measure. Therefore, the enigma is whether HPLC accurately predicts a nontoxin or possible toxic component versus whether ELISA using 15F3.E5 detects the toxin(s) but possibly with insufficient precision and accuracy for clinical or diagnostic value. The research reported herein does not provide a conclusive answer as to which test gives results with the greatest biological relevance; rather, it dictates a need for basic research to determine which of the ergot alkaloids are responsible for livestock toxicosis in *Neotyphodium*-infected grasses, with concomitant effort towards development of sound diagnostic techniques.

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a. Sigma Chemical Co., St. Louis, MO.
b. RBI Research Biochemicals International, Natick, MA.
c. Immulon 4, Dynatech Laboratories, Chantilly, VA.
d. Elx50 Autostrip Washer, Elx808 Ultramicroplate Reader, Bio-Tek Instruments, Winooski, VT.
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Chapter 4

CHARACTERIZATION OF GROWTH AND HEPATIC *IN VITRO* METABOLISM OF ERGOTAMINE IN MOUSE LINES DIVERGENTLY SELECTED FOR RESPONSE TO ENDOPHYTE TOXICITY

Jennifer M. Duringer and A. Morrie Craig

Abstract

This study investigated if genetic divergence exhibited in mice previously selected for susceptibility or resistance to endophyte toxicity had persisted over ten generations of relaxed selection. Experimental groups were endophyte-susceptible or endophyte-resistant mouse lines containing males and females placed on endophyte-free diet (E-) or challenged with an endophyte-infected diet (E+). No differences in litter size and weight gain of pups between the susceptible and resistant mouse lines remained after ten generations of breeding. However, metabolism of a representative ergot alkaloid, ergotamine, using liver microsome preparations from mice presented a more complicated picture. A gender difference was observed in the xenobiotic transformation of ergotamine by liver microsomes from mice on the endophyte-free diet. Females exhibited a greater amount of transformation products 1 (M1 and $M1_i$) and 2 (M2 and $M2_i$) than males in the endophyte-free group. Animals on the endophyte-infected diet showed increased amounts of polar metabolites M3 and M4. Analysis of metabolites by LC-MS confirmed the identity of ergotamine and its isomer in addition to four hydroxylated ergotamine transformation products in the HPLC profiles (M1, $M1_i$, M2 and $M2_i$).

Introduction

Tall fescue (*Festuca arundinacea*) plants are often infected with the endophyte *Neotyphodium coenophialum*, which is a fungus that confers benefits such as drought stress tolerance and pest resistance to the plant, while the plant provides energy and a sustainable environment for the fungus (Adcock et al. 1997). This beneficial symbiotic relationship between the plant and fungus is necessary to ensure optimal plant production and survival. However, the presence of endophyte in the plant results in the production of

ergot alkaloids, which are natural products of *N. coenophialum* shown to be toxic to livestock and are represented by a group of compounds related to lysergic acid (Figure 4.1) (Shelby and Kelley 1991).

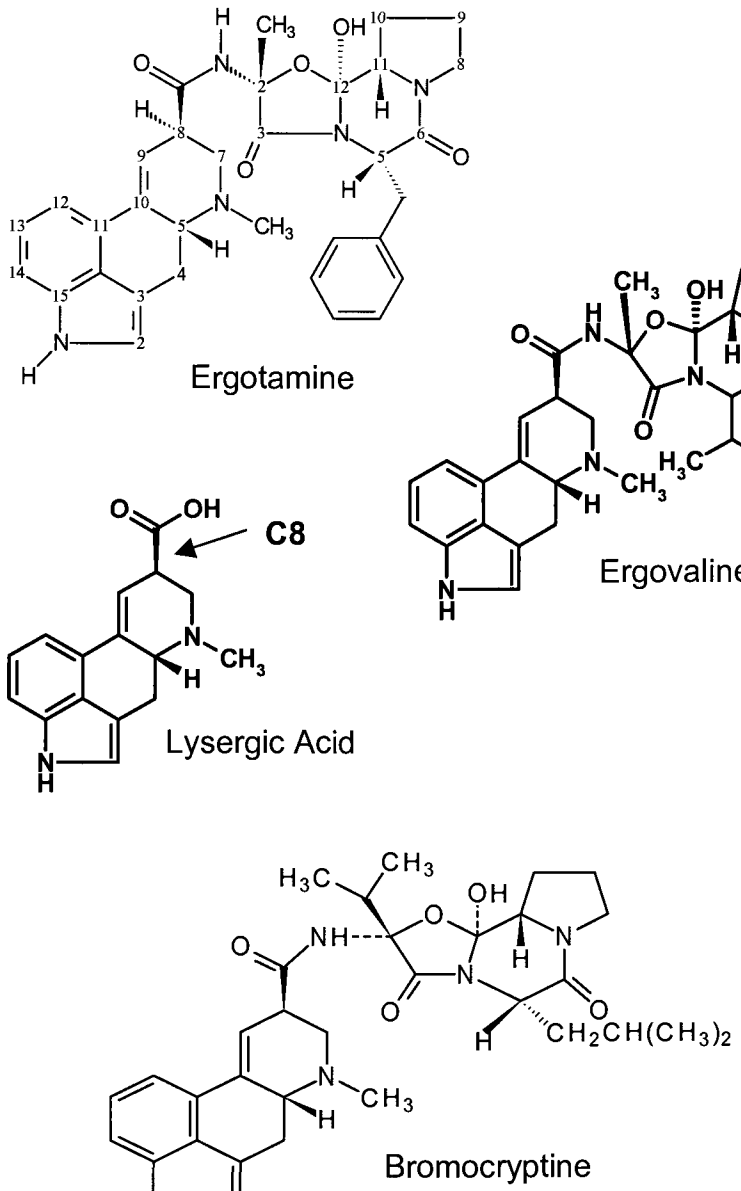


Figure 4.1 Chemical structures of the ergot alkaloids ergotamine and ergovaline and the lysergic acid base of the molecule.

Ergot alkaloids cause over $600 million dollars in livestock damage each year in the United States alone due to the induction of fescue toxicosis (Fribourg et al. 1991). Fescue toxicosis affects animal production by decreasing average daily weight gains and reproductive efficiency. Clinical signs of fescue toxicosis include necrosis and sloughing of extremities as manifested in fescue foot, and other toxic effects exerted after livestock consumption of endophyte-infected plant material (Stuedemann et al. 1998, Craig et al. 1994, Shelby and Kelley 1991). Ergovaline is the predominant ergot alkaloid identified in E+ tall fescue (Yates and Powell 1988, Lyons et al. 1986), and so is targeted in diagnostic testing to determine if plant material is suitable for livestock consumption. However, due to the difficulty in synthesizing large quantities of ergovaline and the high cost of doing so, ergotamine is used as a model compound given its similar chemical structure which suggests parallel toxic effects (Moubarak et al. 1993).

The three disorders associated with consumption of endophyte-infected tall fescue in livestock are summer syndrome, fescue foot and fat necrosis. Clincial signs of 'summer syndrome' or 'summer slump' include poor weight gain, intolerance to heat, excessive salvation, rough hair coat, elevated body temperature, nervousness, low milk production and reduced conception rate (Hoveland 1993, Hoveland et al. 1983). Increased rectal temperature, respiratory rate and heart rate have been observed in cattle grazing E+ fescue (Hemken et al. 1981, Hemken et al. 1979). This may be an adaptive mechanism to dissipate energy, particularly in areas like the southeast where summer temperatures intensify these physiological changes (Thompson and Stuedemann 1993). This disorder is the largest cause of economic loss to livestock producers both because of its wide range of physiological effects on cattle and because cattle from the southern region of the US often bring reduced prices at the market place due to the predominance of E+ fescue in that region (Schmidt and Osborn 1993). Fescue foot is associated with colder temperatures and begins as a red line at the coronary band of the hoof

followed by gangrene, necrosis and even sloughing of the affected hoof (Thompson and Stuedemann 1993). Other affected extremities include the tips of ears and the end of the tail.

Ergot alkaloids act as α-adrenergic agonists, which stimulates smooth muscle cell contraction and results in vasoconstriction (Thompson and Stuedemann 1993). When animals are subjected to extreme temperatures, they have trouble regulating blood flow to their extremities which can result in tissue ischemia and necrosis and sloughing of these body sections in the case of cold weather and an inability to properly cool these body sections in the case of hot weather. Vasoconstriction can even result in strokes in some extreme cases.

Ergot alkaloids also act as dopamine agonists which inhibits the production of prolactin and results in diminished lactation (Oliver 1997, Larson et al. 1994). Prolactin suppression results in reduced milk yield in prepartum cattle but has no effect once lactogenesis occurs (Thompson and Stuedemann 1993). However, lactational yield is a complex balance between hormones and feed intake. Calf birth weights of beef cows on E+ fescue were not affected but milk production was reduced 100 days postpartum by 50% (Schmidt et al. 1986). Holstein cows grazing endophyte-infected ryegrass-fescue hybrids had decreased milk yields compared to cows on endophyte-free grass, most likely due to reduced feed intake for cows on the toxic grass (Hemken et al. 1979). In addition, the rough hair coat seen in cattle experiencing summer slump is due to depressed prolactin levels (Thompson and Stuedemann 1993).

Fat necrosis is manifested by the presence of hard, mesenteric fat surrounding the intestinal tract from the abomasum to the rectum (Bush et al. 1979). These deposits can disrupt digestive processes and interfere with calving or renal function (Thompson and Stuedemann 1993) by causing inadequate space to be available for these functions in the abdominal cavity (Schmidt and Osborn 1993). As part of this syndrome, serum cholesterol

concentration is reduced in cattle and sheep that are exposed to E+ fescue (Stuedemann et al. 1985). This has been interpreted as a response to stress and alters fat metabolism by increasing the saturated fatty acid concentration of fat deposits in steers grazing E+ tall fescue (Thompson and Stuedemann 1993).

Laboratory mice may serve as a model to detect genetic differences between resistant and susceptible animals since they have been shown to have reduced reproductive capacity in pregnancy and litter size (Godfrey et al. 1994, Zavos et al. 1987a, Zavos et al. 1987b) and depressed growth of pups due to reduced suckling performance of the dam (Zavos et al. 1988) when exposed to an E+ diet. Using mice compared to livestock permits the investigation of genetic selection to fescue toxicosis utilizing less monetary resources and experimental time since mice reach reproductive maturity within two months, have a high fecundity, and environmental conditions can be easily controlled. A long-term goal of this research has been to identify biochemical parameters that differ between the lines which could be applied to livestock to measure susceptibility amongst individuals, herds, breeds and species. Livestock could then be preferentially selected for breeding using these physiological biomarkers in order to confer greater resistance to endophyte toxicity within a herd.

In New Zealand, for example, the *Pithomyces chartarum* fungus in perennial ryegrass produces sporidesmin which causes occlusive damage to the bile duct system and results in secondary photosensitization manifested as facial eczema (Hohenboken et al. 2000). After selecting rams that exhibited sporidesmin resistance, the impact of facial eczema was reduced in animals exposed to this toxin (Morris et al. 1995). A mouse line for endophyte-susceptibility and resistance was used as a possible model for general resistance to toxins. It was found that upon exposure to sporidesmin, endophyte-resistant mice were more resistant to liver and kidney lesions than were the endophyte-susceptible mice (Hohenboken et al. 2000). This

presented the possibility for selecting livestock that are resistant to multiple toxins, creating an invaluable resource to livestock producers concerned about multiple toxin exposures in rangelands. However, this requires further work on the identification of a easily measured bioindicator of toxin resistance that is inherited and non-invasive (Hohenboken et al. 2000) and responds to a wide array of toxins.

The experiments performed in this study are a continuation of research where mice with a measurable genetic divergence in susceptibility to endophyte toxicity served as a model for studying the physiological effects and metabolism of ergot alkaloids (Wagner et al. 2000, Hohenboken and Blodgett 1997, Miller et al. 1994). The initial selection for divergence in response to tall fescue toxicosis was carried out for eight generations over a period of approximately two years by Hohenboken and Blodgett (1997). Male and female ICR (CD-1®) outbred mice from Harlan Sprague Dawley (Indianapolis, IN) were used in these experiments. Mice were mated at eight weeks of age. Litters were weaned at 21 days of age. This cycle was repeated for eight generations. Selection for resistant and susceptible parents was made four weeks after weaning, as detailed below. Response to the effects of feeding endophyte infected diets were determined by comparison of pup growth rate and suckling behavior.

Individual mice were tested for the toxicosis response by quantifying the reduction in their growth rate when E+ fescue seed was present in the diet, compared to their growth rate when E- fescue seed composed the same proportion of the diet. Mice were weighed individually at weaning and four same-sex non-sibling mice were assigned to a cage. Mice were provided a diet of 50% commercial rodent food and 50% E- tall fescue seed *ad libitum* for two weeks. For the next two weeks, mice were fed a diet of 50% commercial rodent food and 50% E+ tall fescue seed *ad libitum*. A control group of one male and female from each litter were maintained on the E- diet for the entire

four weeks. Mice were weighed once a week and fresh food was provided three times per week.

Average daily gains (ADG) of each mouse during the E- and E+ diets were computed as the linear regression coefficient of weight on days on feed. The ADG during E- feeding was computed from weights taken at weaning and at 1 and 2 weeks of the experiment. ADG during E+ feeding was computed from weights recorded at 2, 3 and 4 weeks of the experiment. These values were entered into the following formula which measures the percent reduction in ADG associated with E+ feeding:

$$R_M = [[(ADG\ E-) - (ADG\ E+)]/(ADG\ E-)] \times 100$$

The average mouse would have grown more slowly the latter two weeks of the test, even if the diet had not been changed. The effects of advancing age and the E+ diet were partitioned by using the ADG data from the mice maintained on the E- diet for the entire four weeks. In addition to R_M, a probable breeding value (PBV) for toxicosis response of each tested mouse was computed from the mouse's R_M (percent reduction in ADG) and from the average R_M of its litter. This was done to increase the accuracy of breeding value estimation and rate of response to selection and was calculated as follows:

$$PBV = [(1-r)/(1-t) \times (R_M - R_L)] + [((1 + (n - 1)r)/(1 + (n - 1)) \times (R_L - R_P)]$$

where r = the genetic relationship (0.50) among littermates, t = the observed intraclass correlation among littermates for R_M and R_P = the population average R_M of all tested mice in the resistant or susceptible line for that generation.

Within each line, mice of each sex were ranked for PBV. To continue the resistant line, males and females were selected that had the largest negative PBV. These mice would have the smallest reduction in ADG during the E+ experimental period. To continue the susceptible line, animals with the largest positive PBV were selected. To reduce inbreeding, whenever possible no more than two animals per sex were chosen from any one litter.

Earlier work done with these mice showed higher levels of the phase II glutathione-*S*-transferase (GST) and UDP-glucuronosyltransferase (UDPGT) enzymes in the resistant line but no differences in total cytochrome P450 levels (Hohenboken and Blodgett 1997). No characterization of ergot alkaloid metabolism by liver microsomes was reported. A recent study found that resistant mice countered the toxic effects of an endophyte-infected seed diet better than susceptible mice, primarily by increased weight gain soon after maturation and larger litter size (Wagner et al. 2000). They also found low correlation between GST and UDPGT activity and reproductive traits, suggesting that the physiological mechanism imparting resistance to endophyte toxicity is something other than these Phase II enzymes.

The objectives of this study were: 1) to determine whether the genetic divergence established in previous generations of the endophyte-resistant mouse line had persisted over ten generations of relaxed selection, 2) to characterize the metabolism of the ergot alkaloid ergotamine in mouse liver microsomes from both lines as a measure of the oxidative pathway of detoxification before and after exposure to endophyte-infected feed to see if differences in susceptibility to endophyte toxicosis is due to differences in metabolism and 3) to identify the major hepatic transformation products of ergotamine by mass spectrometry.

Materials and Methods

Chemicals and materials

Chemicals. Potassium phosphate, ergotamine tartrate, ethylenediamine tetraacetic acid (EDTA), dithothreitol (DTT), phenylmethyl sulfonyl fluoride (PMSF), Trizma base, glycerol, bovine serum albumin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride and nicotinamide adenine dinucelotide phosphate (NADPH) were obtained from Sigma

Chemical Company (St. Louis, MO). Sodium hydroxide, potassium chloride, acetic acid and HPLC grade solvents (acetone, acetonitrile, methanol and chloroform) were purchased from Fisher Chemicals (Fairlawn, NJ). Ammonium carbonate was obtained from Aldrich (Milwaukee, WI). Ergosil™ silica gel was purchased from Analtech (Newark, DE). Coomassie reagent was obtained from Pierce (Rockford, IL). Sodium sulfate was purchased form Mallinckrodt (Paris, KY). Water was from an ion-exchange reverse osmosis membrane filtration system. Filter paper discs were purchased from Schleicher and Schuell (Riviera Beach, FL).

HPLC system. The HPLC system consisted of a Perkin Elmer (Shelton, CT) series 200 autosampler fitted with a 20 µl sample loop and a Perkin Elmer LC 250 solvent pump linked to a Perkin Elmer LC240 fluorometer or to a Perkin Elmer 235C photodiode array detector.

Analysis of endophyte-free and endophyte-infected tall fescue seed

Samples were prepared for analysis of ergovaline according to a previously reported method (Craig et al. 1994). Five random handfuls of endophyte-free (E-) and endophyte-infected (E+) fescue seed were sampled from their containers and pooled. The contents were ground in a Cyclotec 1093 sample mill grinder (Tecatur, Sweden) with a 0.5 mm screen in a fume hood. A 1.1 mg/ml ergotamine tartrate in methanol internal standard stock solution was made weekly. This solution was diluted with chloroform to 1 µg/ml just prior to the extraction procedure. Reference material was prepared by mixing ground endophyte-free fescue seed with highly infected ground fescue seed of a known concentration to produce five concentrations of 526, 750, 1500, 2300 and 3000 ppb ergovaline used to generate a standard curve for each run. The reference material levels were quantified a minimum of twenty times against current reference material of known ergovaline concentration. A positive control was run with each analysis as well for quality control purposes (2115 ppb ergovaline). The positive control was ground seed that had been tested

for ergovaline concentration 20 times using the five known reference materials.

Samples were extracted as follows: one gram of control, sample or reference material was weighed into a screw cap centrifuge tube and then 10 ml chloroform, 1 ml of 1 µg/ml ergotamine tartrate internal standard solution and 1 ml of 1 mM sodium hydroxide was added in a fume hood. The tubes were allowed to turn for 24 hours in the dark on a Fisher Rotorak. The samples were then centrifuged at 2000*g* for 5 minutes. Extracts were purified on Ergosil™ solid phase extraction cartridges assited by vacuum elution. The cartridges were prepared in 6 ml monoject disposable syringes by layering a 12.5 mm filter paper disc, then one gram of compacted Ergosil™ silica gel, another filter disc, 0.5 g compacted anhydrous sodium sulfate topped by a filter disc. The SPE columns were conditioned with 5 ml chloroform prior to introduction of the sample. A volume of 4 ml of chloroform was added to each column. Supernatant from the centrifuged samples was added to the top of the syringes and allowed to pass through the column. Two 1 ml aliquots of a chloroform/acetone (75/25 v/v) mixture and one 1.5 ml aliquot of methanol were added next. After complete elution of these washes, test tubes were placed under the SPE columns. The ergopeptides were eluted with one 2.5 ml aliquot of methanol which was caught in the test tubes and dried under N_2 gas at 50°C (N-Evap 112 Organomatron Associates Inc. Berlin, MA) in a fume hood. The sample was reconstituted with 500 µl methanol, sonicated for 10 seconds and centrifuged for 5 minutes at 2000 rpm. Samples were transferred to amber autosampler vials to avoid exposure of the ergovaline to light.

Ergovaline was detected on the HPLC system by fluorescence at λ_{ex} = 250 nm and λ_{em} = 420 nm. Separation was performed on a Jordi RP SM-500 polymeric divinyl benzene (5 µm particle size) column (Alltech, Deerfield IL) with a guard column of similar packing (7.5 x 4.6 mm). Acetonitrile and 2.6

mM ammonium carbonate (70/30 v/v) separated the samples isocratically at a flow of 1.0 ml/min. Samples were compared to a standard curve from reference seed samples run with each analysis. They were plotted as the ergovaline/ergotamine height ratio versus the concentration of ergovaline.

Mating and selection of mice

Lines of endophyte-resistant and –susceptible mice were established previously in male and female ICR mice from Harlan Sprague Dawley (Madison, WI) (Hohenboken and Blodgett 1997). Selection for response to endophyte toxicity was discussed above and involved quantifying the reduction in growth rate and probable breeding value when E+ fescue seed was present in the diet, compared to the growth rate and probable breeding value when E- fescue seed was fed. Experiments were performed on these mice after establishment of the two genetically divergent lines and usually involved a four-week postweaning test (2 weeks on E- feed, 2 weeks on E+ feed) to select mice for continuation of the lines (Hohenboken et al. 2000, Wagner et al. 2000, Hohenboken and Blodgett 1997). Unfortunately, the amount of ergovaline or other ergot alkaloids was measured in only one of the experiments in these studies, so a detailed history of ergot alkaloid exposure to these mice lines is unavailable. Mice were mated through relaxed selection for ten generations where resistant and susceptible lines remained distinct but no effort was made to mate the most resistant female with the most resistant male or to mate the most susceptible female with the most susceptible male. For the ten generations of relaxed selection before our experiment, mice were provided commercial rodent chow (Harlan Teklad 2018, Harlan Sprague Dawley) *ad libitum*.

All selection, breeding and feeding experiments were carried out in the Laboratory Animal Vivarium at Virginia State and Polytechnic University (Blacksburg, VA) under conditions similar to those in Hohenboken et al., (1997). All procedures were approved by an IACUC committee at Virginia State and Polytechnic University. Water was provided by continuous flow

nipple waterers *ad libitum*. Feed for this experiment is discussed below. All animal cages were housed in a single environmentally controlled room at an average temperature of 24°C with a 12 hour light and 12 hour dark cycle. Three mice/cage were housed in 15 cm x 21 cm x 20 cm transparent plastic cages. Pine wood shavings were used for bedding. The experimental design for mouse selection in the present experiment is shown in Figure 4.2 for each line.

Forty female mice each of the resistant and susceptible lines (Hohenboken and Blodgett 1997) were mated to a single male from their same line over 5 days. At the end of mating, male mice were removed and killed by carbon dioxide gas inhalation. Within 2 days following parturition, each litter with over 11 offspring was reduced to 11 by killing randomly chosen excess pups via

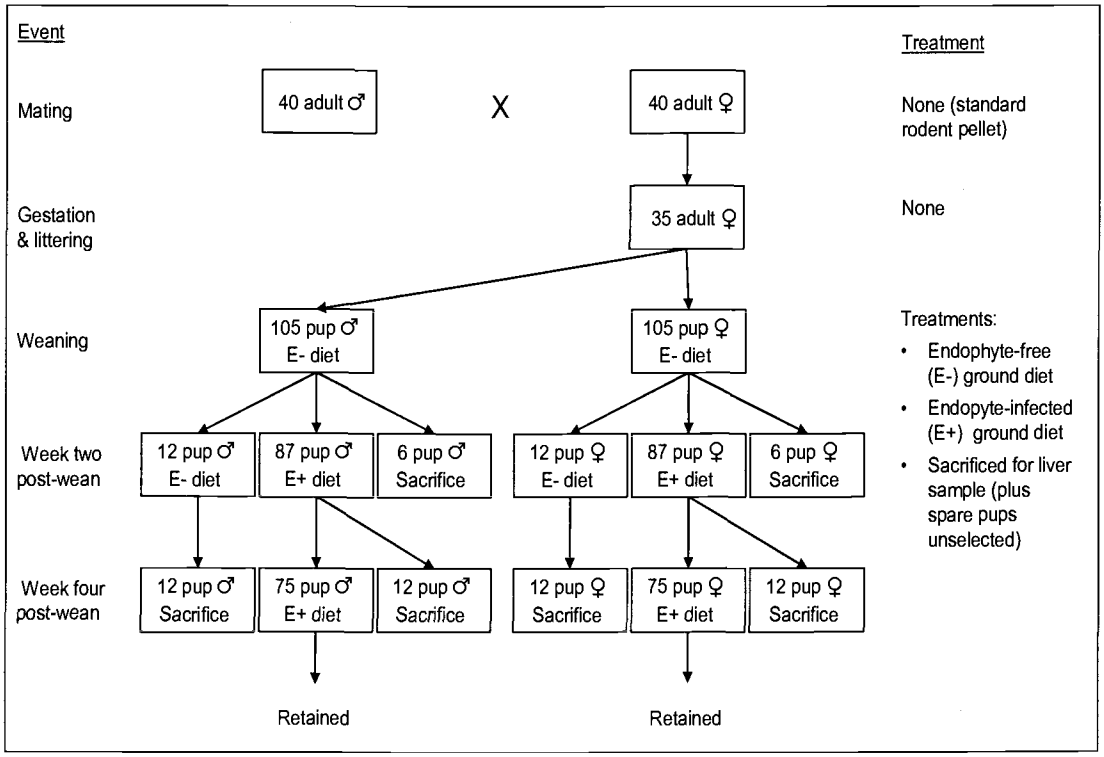


Figure 4.2 The experimental groups and treatments used in the experiment for one line of mice (i.e. susceptible or resistant line). The experimental layout was identical for both lines.

cervical dislocation. Females that failed to litter were killed by gas inhalation. Mice were weaned once most litters reached 21-days of age and the feedings treatments began. At weaning, the pups were sexed and weighed, with three pups of the same sex and line randomly chosen from the separate litters and co-housed in a standard cage. There were 35 cages (105 mice) per line-sex combination. All remaining females and any excess pups were killed by carbon dioxide gas inhalation.

Endophyte-infected tall fescue feeding experiment

The weaned mice were fed a ground and mixed diet in ceramic or metal bowls, with feed replaced three days a week during the experiment. Sufficient food was provided to allow *ad libitum* intake. For the first 2 weeks, all mice were fed a diet composed of 50% endophyte-free ground fescue seed and 50% (w/w) of Harland Teklad (chow 2018) ground rodent food. At the end of this two week period, two cages (six mice) were chosen at random from within each line-sex combination and killed by cervical dislocation and their livers were removed. Four cages per line-sex combination (12 mice) were chosen at random and continued to receive the E- diet for the next 2 weeks. The remaining 29 cages per line-sex combination (87 mice) were switched to a diet of 1381 ppb ergovaline (50/50 w/w E+ fescue seed and ground rodent food).

At the end of the feeding treatment, mice in all 4 cages of each line-sex combination on the E- diet were sacrificed and their liver was removed. In addition, 4 cages per line-sex combination from the E+ treatment were randomly chosen and sacrificed for liver collection. The remaining mice on the E+ diet were retained to maintain the colony. Mice were weighed weekly throughout the four week feeding experiment.

Liver preparation and storage

A diagram of the mice sacrificed for liver removal is displayed in Figure 4.3. After euthanizing the mice by cervical dislocation, the liver was immediately excised and cut into approximately ½-inch pieces. All liver pieces (0.82-2.13 g) from an individual mouse were wrapped twice in aluminum foil, labeled

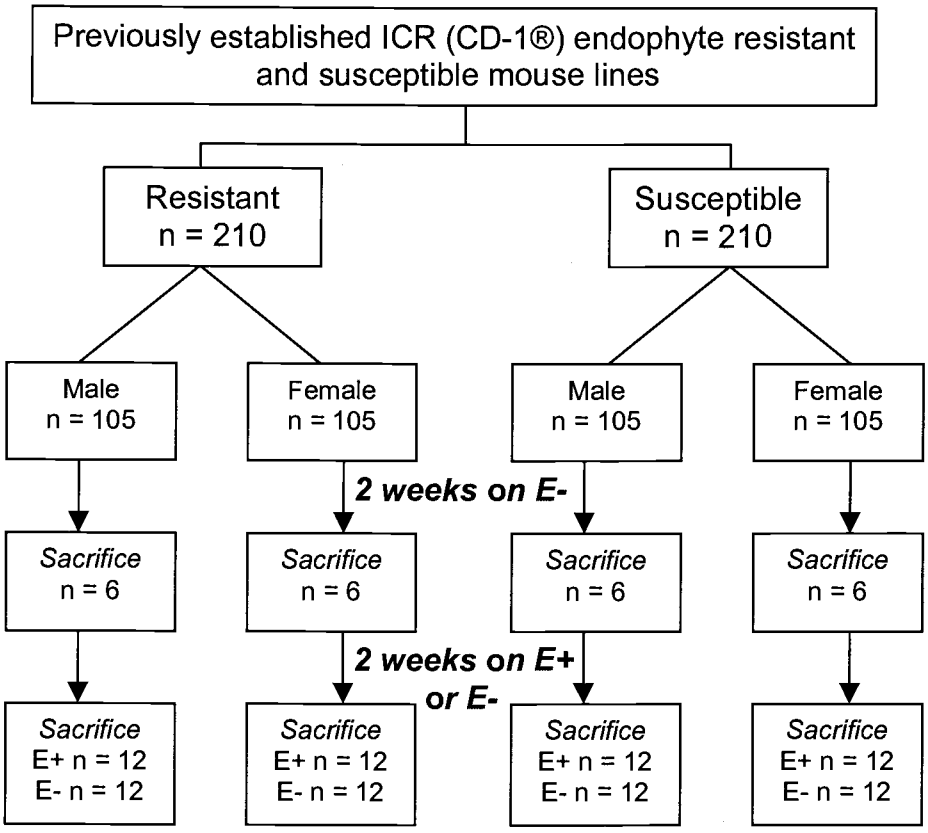


Figure 4.3 Layout of mouse sacrifice from all line-sex combinations for making liver microsomes. Six mice per line-sex group were sacrificed after the first two weeks on the E- diet for a total of 24 mice. Twelve mice per line-sex-diet group were sacrificed after the second two weeks for a total of 96 mice. The remaining 75 mice from each line-sex group were retained to maintain the colony.

(animal identification, line, sex, diet, date) and placed in a filled liquid nitrogen dewar. The samples were then shipped overnight to the Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University.

Liver microsomes were made as soon as possible after arrival, according to a previously reported protocol (Huan et al. 1998). The liver tissue was placed in 4 ml of homogenization buffer (final concentration of 0.1 M Tris acetate (pH 7.5), 0.1 M KCl, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF) per gram of original tissue and homogenized using a Potter-Elvjhem homogenizer in an ice bath. The suspension was centrifuged at 10,000*g* for 30 minutes at 4°C in a

refrigerated Sorvall centrifuge. The supernatant was then centrifuged at 105,000*g* for 60 minutes and the centrifuged supernatant was saved as the cytosol fraction and frozen at -80°C until used. The microsomal pellet was resuspended with a Potter-Elvjhem homogenizer in 1 ml of resuspension buffer (final concentration of 10 mM Tris acetate (pH 7.4), 0.1 mM EDTA, 20% glycerol, 1 mM DTT and 0.1 mM PMSF) per gram of original tissue and was stored in aliquots at -80°C.

Protein concentration determination

Microsomal and cytosolic protein content were determined with bovine serum albumin as the standard as described previously (Lowry et al. 1951). Microsomes were diluted 1:100 in H_2O. A volume of 100 µl of the diluted sample, standard (50, 100, 200 and 300 µg/ml bovine serum albumin) or blank (H_2O) were added to test tubes in triplicate. One ml of Coomassie Reagent was added to the test tubes and mixed with a vortex mixer. Into a 96-well plate was dispensed 200 µl of each reaction mixture. The absorbance was measured at 595 nm using a spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA). The protein concentration was calculated from the standard curve produced from the bovine serum albumin standards.

In vitro *metabolism of ergotamine (ET) by liver microsomes*

Following the protocol of Moubarak and Rosenkrans (2000), microsomal incubations consisted of a final concentration of 0.5 mg protein, 100 mM Tris-HCl, 10 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 20% glycerol, and 1.0 µg/ml ergotamine in a total volume of 0.5 ml. The mixture was equilibrated for 5 minutes at 37°C in a shaking metabolic incubator set at 100 cycles/minute. The reaction was initiated with addition of the NADPH-generating system (66 mM glucose-6-phosphate, 2.0 unit/ml glucose-6-phosphate dehydrogenase, 66 mM magnesium chloride and 26 mM NADPH) and incubated for 30 minutes. The reaction was terminated by addition of 100 µl of 94% acetonitrile/6% glacial acetic acid. The mixture was then vortexed

and centrifuged at 12,000*g* for 15 minutes. An aliquot of the supernatant was removed for HPLC analysis.

HPLC analysis of ergotamine and its metabolites

Ergotamine and the extent of biotransformation products produced during incubations with mouse liver microsomes was monitored by reverse phase HPLC with an Upchurch C18 guard column and a Luna C18 column (250 x 4.6 mm, 5 µm particle size (Phenomenex (Torrance, CA)) at ambient temperature. The metabolites were detected by fluorescence at λ_{ex} = 250 nm and λ_{em} = 420 nm or by UV at λ = 254 nm. Data was captured using an analogue-to-digital converter interfaced to a computer with Turbochrom software (Perkin Elmer). Column elution was performed with 0.1 M ammonium carbonate (A) and acetonitrile (B) as follows:

Time	Flow rate (ml/min)	A	B
0	1.0	95%	5%
1	1.0	95%	5%
46	1.0	0%	100%
51	1.0	0%	100%
56	1.0	95%	5%
60	1.0	95%	5%

Solid phase extraction of ergotamine and its metabolites

A C18 solid phase extraction cartridge (C18 Sep-Pak, 500 mg, Waters Corp, Milford, MA) attached to a SPE vacuum manifold was first conditioned with 6 ml of methanol followed by 12 ml of 0.1M ammonium carbonate. Approximately 15 ml of centrifuged supernatant obtained from the *in vitro* metabolism of mice was pooled and added to the SPE column and washed with 1 ml of 0.1 M ammonium carbonate. The SPE cartridge was then sequentially eluted with 3 ml each of 20%, 40%, 60%, 80% and 100% acetonitrile (ACN) in H_2O into five separate tubes. The column was allowed to run dry after addition of 100% ACN. The collected fractions were concentrated to dryness under N_2 gas and reconstituted in 100 µl methanol.

These fractions were then analyzed according to the HPLC procedure previously described for ergotamine and its metabolites.

Fraction collection of ergotamine and its metabolites

The 40% and 60% ACN fractions had the largest concentration of ergotamine and its metabolites and were therefore selected for fraction collection. These two samples were pooled and separated by HPLC using the photodiode array detector with manual collection of six peaks ergotamine, metabolite 1, metabolite 2, ergotamine epimer, metabolite 1 epimer and metabolite 2 epimer into six separate vials. A volume of 25 µl methanol was added to the remaining sample and injected in order to recover as much of the metabolites as possible. These six fractions were then dried down under N_2 gas, reconstituted in 1 ml methanol, checked for purity via photodiode array and fluorescence detection and stored at -20°C until LC/MS analysis.

LC/MS analysis of ergotamine and its metabolites

Spectra were obtained on a Thermo Finnigan (San Jose, CA) LCQ Classic ion trap mass spectrometer equipped with a custom designed electrospray inlet consisting of a 30 micron i.d. steel capillary heated at 160°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.

Statistics

Average daily gains (ADG) of each mouse during the E- and E+ diets were computed as the linear regression coefficient of weight on days on feed, as previously described (Hohenboken and Blodgett 1997). The ADG during E- feeding was computed from weights taken at weaning and at 1 and 2 weeks of the experiment. ADG during E+ feeding was computed from weights recorded

at 2, 3 and 4 weeks of the experiment. Least-squares means were means calculated for the responses (ADG and the individual metabolites) assuming a normal distribution.

The experiment used a split-plot design defining the experimental unit as an individual cage. Each cage was assigned a feed treatment for the feed challenge period and had mice of a given line and sex. Each cage contained three mice and each mouse was assayed in duplicate for the liver microsome incubation assay. Therefore, nested within cage is mouse and nested within mouse is the duplicate measure of ergotamine and its metabolites. The error term for testing the treatment effects (feed, line and sex) is the cage. The random terms were cage, mouse within cage and replicate mouse within cage.

A model that fit feed, line and sex as the main effects and their two-way interactions (line*sex, line*feed and sex*feed) as the fixed effects was used to perform ANOVA on the response variables (average daily gain or the individual metabolites formed in the ergotamine metabolism experiments). These models were used to analyze for significant differences ($p < 0.05$) between mouse lines and between genders within the same line for the response variables. The feed term was only applicable during the second two weeks when mice were on an E+ or an E- diet. Thus, the model for the first two weeks is represented as $\mu\{response \mid line, sex\} = line + sex + line*sex$. The model for the second to weeks is represented as $\mu\{response \mid line, sex, feed\} = line + sex + feed + line*sex + line*feed + sex*feed$.

Results

Analysis of endophyte-free and endophyte-infected tall fescue feed

The endophyte-free seed had no detectable ergovaline and the endophyte-infected seed had 2761 ppb ergovaline when assayed by HPLC. When mixed in the 50/50 w/w rodent chow/E+ seed ratio fed to the mice, a concentration of approximately 1381 ppb ergovaline is calculated to be in the E+ diet.

Concentrations of 200 ppb ergovaline for cattle and 500-1000 ppb ergovaline for sheep have been correlated to clinical symptoms of fescue toxicosis (Debassai et al. 1993)). The dose used in this study is therefore a relatively high exposure dose.

Endophyte-infected tall fescue feeding experiment

I. Weight gain analysis

Mice were fed a diet of endophyte-free food for the first two weeks. The average daily gain during this period is summarized in Table 4.1. A difference in average daily gain (ADG) was evident between both lines ($p < 0.01$) and genders ($p < 0.01$). Resistant males had an ADG of 0.65 while susceptible males had an ADG of 0.77, a difference of 0.12 g/day. Resistant females had an ADG of 0.45 while susceptible males had an ADG of 0.55, a difference of 0.10 g/day. Resistant males had an ADG of 0.65 while females had an ADG

	Average Daily Gain (g/day)	
Line-Gender Groups	**First Two Weeks**	**Second Two Weeks**
Resistant Females	0.45	0.13
Susceptible Females	0.55	0.11
Resistant Males	0.65	0.26
Susceptible Males	0.77	0.27

Table 4.1 Least squares means for average daily gains (grams/day) for mice as measured from weekly weights taken during the experimental period. Values are given in grams/day, n = 6 for each group during the first two weeks; n = 12 for each group during the second two weeks. The first two weeks corresponds to a diet of 50/50 rat chow/endophyte-free seed after weaning. The second two weeks corresponds to a diet of 50/50 rat chow/endophyte-infected seed of 1381 ppb ergovaline (E+) or a diet of 50/50 rat chow/endophyte-free seed (E-) fed during the second two weeks.

of 0.45, a difference of 0.20 g/day. Susceptible males had an ADG of 0.77 while females had an ADG of 0.55, a difference of 0.22 g/day.

The next two weeks mice were changed to an E+ diet consisting of 1381 ppb ergovaline or continued on the E- diet. The ADG during this period is summarized in Tables 4.1 and 4.2. Differences in growth rate after challenge with endophyte-infected feed observed previously between these lines of mice was not evident in the current study. After consuming an E+ diet for two weeks, resistant mice had a slightly lower ADG than susceptible mice (0.16 g/day versus 0.18 g/day) (Table 4.2). Resistant and susceptible males exhibited nearly identical ADG values at 0.26 and 0.27 g/day, respectively (Table 4.1). Resistant females showed a slightly larger growth rate during the second two weeks on the E+ diet at 0.13 g/day while susceptible females had an ADG of 0.11 g/day. Gender differences in ADG were maintained during the second two weeks of the experimental period ($p < 0.01$). Males maintained a larger ADG than females in both lines. Resistant males gained an average of 0.26 g/day while resistant females gained 0.13 g/day. Susceptible males

Line-Diet groups	**Average daily gain** (g/day)
Resistant E+	0.16
Susceptible E+	0.18
Resistant E-	0.23
Susceptible E-	0.19

Table 4.2 Least squares means for average daily gains (grams/day) for mice as measured from weekly weights taken during the second two weeks of the experimental period. Values are given in grams/day, n = 12 for each group. Line-diet groups correspond to males and females of the same line pooled on a diet of 50/50 rat chow/endophyte-infected seed of 1381 ppb ergovaline (E+) or a diet of 50/50 rat chow/endophyte-free seed (E-) fed after the first two weeks.

gained an average of 0.27 g/day while susceptible females had an ADG of 0.11 g/day.

Feed did not significantly affect ADG during the second two weeks (Table 4.2). Exposure to the endophyte-free diet resulted in a slightly larger weight gain for resistant mice (0.23 g/day) than susceptible mice (0.19 g/day). Endophyte-infected feed negatively affected weight gain in resistant mice as they gained 0.16 g/day compared to susceptible mice who gained 0.18 g/day.

Endophyte-infected tall fescue feeding experiment

II. In vitro *metabolism of ergotamine by liver microsomes*

Hepatic microsome and cytosol fractions were prepared from animals on E+ and E- diets as depicted in Figure 4.3. Hepatic microsome incubations from sixty-eight representative animals were performed with ergotamine to evaluate transformation products by HPLC to see if differences in the metabolism of this representative ergot alkaloid existed between the lines. Ergotamine was chosen to study microsomal metabolism of ergot alkaloids based on its availability and cost compared to ergovaline. Examples of the chromatograms showing the *in vitro* metabolism of ergotamine to its biotransformation products in mouse liver microsomes is shown in Figure 4.4. The bottom four panels of Figure 4.4 shows formation of ergotamine and its transformation products from representative samples of resistant and susceptible males and resistant and susceptible females.

For the first two weeks when all mice were on the E- diet, the only consistently significant effect was the mouse's sex (Table 4.3). Females had higher levels of metabolites 1 and 3-5 and metabolite 1 epimer ($p < 0.050$), lower levels of ergotamine and its epimer but had similar concentrations of metabolite 2 and metabolite 2 epimer ($p > 0.050$). No differences were apparent between resistant and susceptible mice for any of the compounds measured, except for metabolite 3 which exhibited a suggestive but inconclusive difference between the lines ($p = 0.062$).

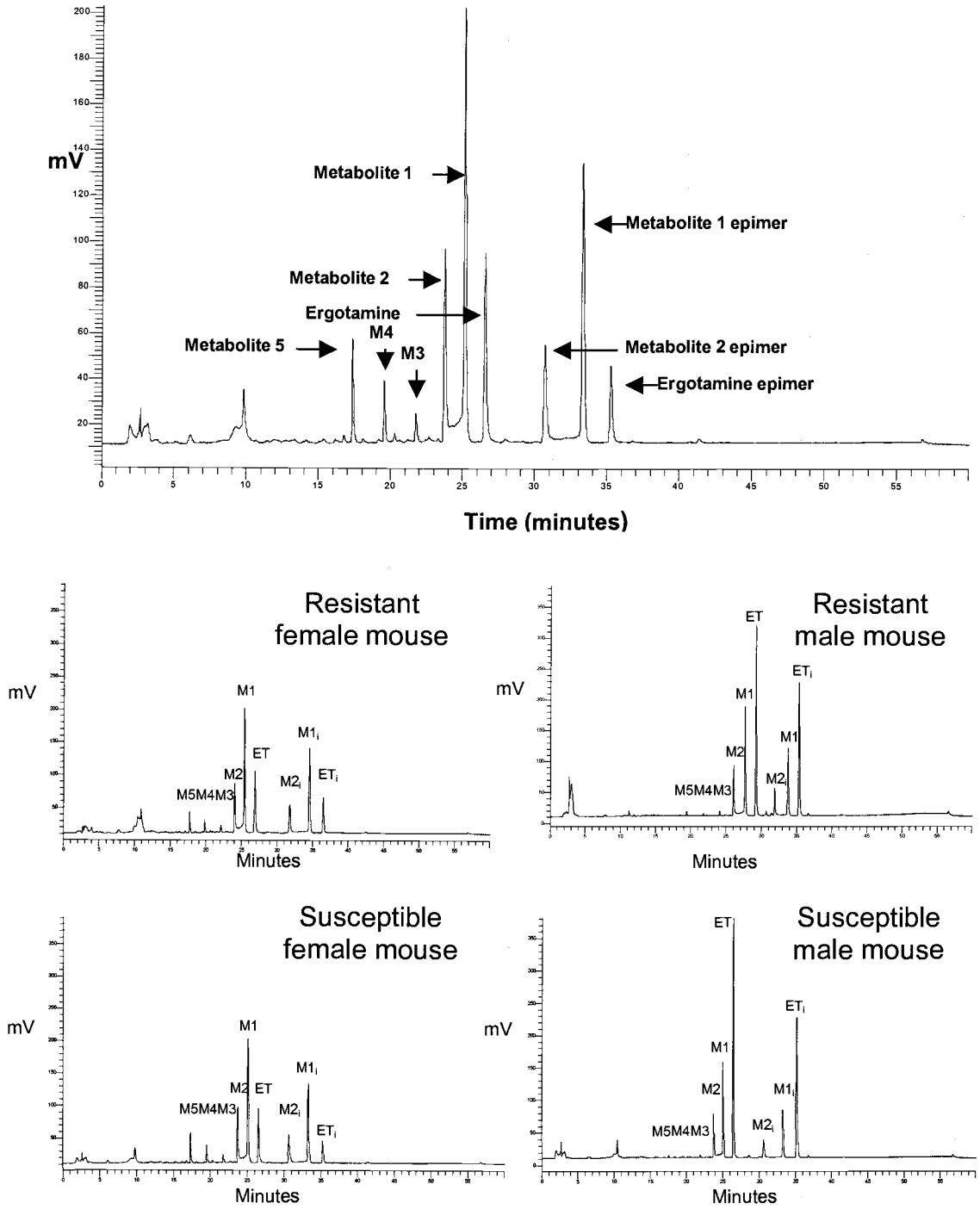


Figure 4.4 Chromatograms of *in vitro* incubation with ergotamine in mouse liver microsomes detected by fluorescence. The top panel is an enlarged chromatogram. The four groups of mice used in the experiment are represented in the bottom four panels: resistant male and female mice and susceptible male and female mice. ET = ergotamine, M1 = metabolite 1 and so forth. $_i$ = epimer.

Table 4.3 Ergotamine and its transformation products from microsomal incubations performed on mice fed an E- diet for two weeks.[†]

	Metabolite identification								
	M1	M2	M3	M4	M5	ET	$M1_i$	$M2_i$	ET_i
Resistant Males	495212 ±3114	778958 ±5914	73699 ±117	40467 ±5207	82925 ±10305	2190596 ±134819	1198420 ±12966	491787 ±8573	1861949 ±85109
Resistant Females	1937742 ±30767	826539 ±19180	104597 ±3008	123314 ±23074	220519 ±31749	1226321 ±147192	1439719 ±16026	570299 ±4446	789751 ±212191
Susceptible Males	1508350 ±128244	676252 ±62755	59909 ±9793	37800 ±13769	66857 ±20479	2376724 ±352875	1118994 ±110439	431592 ±48436	1718177 ±236008
Susceptible Females	1845244 ±111200	761261 ±55646	88935 ±8153	100356 ±18263	161733 ±26099	1430870 ±212686	1414850 ±78886	541850 ±31356	700571 ±150970

[†]Values are given as peak area ± SE of the metabolite as determined by HPLC with fluorescence detection. Five mice were analyzed in duplicate for each of the four groups. ET= ergotamine, M1 = metabolite 1, M2 = metabolite 2 and so forth. $_i$ = epimer of that metabolite.

For the second two weeks when the E+ feed was introduced to some of the mice, the metabolism of ergotamine in each of the groups presented a more complicated picture (Tables 4.4 and 4.5). Metabolite 1 showed no significant differences. Metabolite 2 showed a suggestive but inconclusive difference for line*sex only ($p = 0.061$). Metabolite 3 displayed significant differences ($p < 0.050$) for line, sex and line*sex. Metabolite 4 showed significant differences for sex and line*sex and suggestive differences for line ($p = 0.064$) and feed*sex ($p = 0.056$). Metabolite 5 displayed significant differences for sex and suggestive differences for line*sex ($p = 0.055$). Ergotamine showed significant differences for sex only. Metabolite 1 epimer displayed no significant differences. Metabolite 2 epimer showed a significant difference for line*sex. Ergotamine epimer showed a significant difference for sex.

Identification of major HPLC peaks from in vitro *ergotamine metabolism by mouse liver microsomes*

A flow diagram of this process is shown in Figure 4.5.

I. Separation by solid phase extraction

The photodiode array is less selective than the fluorescence detector in that it displays more of the components present in a fraction (Figure 4.6). This is important in terms of the purity of ergotamine and its metabolites collected for mass spectral analysis. Thus, the photodiode array was used as a more discriminating tool in checking for the purity of a fraction. The fluorescence chromatograms of the initial fractions as isolated by the C18 SPE column are shown in Figure 4.7. The 40% and 60% acetonitrile (ACN) fractions were chosen for further purification of ergotamine and its metabolites since they showed the largest concentrations of these compounds.

II. Fraction collection by preparative HPLC

The 40% and 60% ACN fractions were monitored by reverse phase HPLC using UV detection. Ergotamine, ergotamine epimer, metabolite 1, metabolite 1 epimer, metabolite 2 and metabolite 2 epimer peaks were collected

Table 4.4 Ergotamine and its transformation products from microsomal incubations performed on mice fed an E- diet for two weeks followed by an E+ diet for two weeks.[†]

	Metabolite identification								
	M1	M2	M3	M4	M5	ET	$M1_i$	$M2_i$	ET_i
Resistant Males	1622474 ±85015	699148 ±39450	67294 ±7053	43945 ±8780	80411 ±19834	2004800 ±201372	1191764 ±53896	491253 ±23311	1576400 ±128535
Resistant Females	1513081 ±56231	588734 ±17773	134791 ±23333	116922 ±16057	178142 ±23542	1055959 ±124311	1200429 ±40080	422341 ±12979	788657 ±97560
Susceptible Males	1278840 ±228220	681868 ±38615	57773 ±4410	37301 ±6621	68954 ±11051	2226861 ±198414	2519707 ±1317676	458939 ±26363	1713539 ±136533
Susceptible Females	1739822 ±60999	689903 ±26816	98376 ±3400	206967 ±32130	339168 ±113243	1173280 ±186791	1370584 ±41302	477805 ±20194	1021471 ±105564

[†]Values are given as peak area ± SE of the metabolite as determined by HPLC with fluorescence detection. Six mice were analyzed in duplicate for each of the four groups. ET= ergotamine, M1 = metabolite 1, M2 = metabolite 2 and so forth. $_i$ = epimer of that metabolite.

Table 4.5 Ergotamine and its transformation products from microsomal incubations performed on mice fed an E- diet for the entire four week experimental period.[†]

	Metabolite identification								
	M1	M2	M3	M4	M5	ET	$M1_i$	$M2_i$	ET_i
Resistant Males	1678284 ±60852	744294 ±19685	70947 ±3536	48341 ±9192	79692 ±8987	2070684 ±194899	1207575 ±53731	502397 ±18756	1715581 ±182980
Resistant Females	1583414 ±49894	654148 ±20608	160048 ±20099	95036 ±12843	211458 ±17380	892511 ±66949	1144474 ±49129	424413 ±8268	829950 ±86744
Susceptible Males	1486730 ±73739	668024 ±80694	68066 ±16323	40803 ±9233	61744 ±12656	2150659 ±277416	1113230 ±86292	421519 ±28161	1830688 ±142741
Susceptible Females	1450937 ±264899	690359 ±47043	92148 ±6483	125786 ±13191	238355 ±36381	1527896 ±142585	1363784 ±71137	478014 ±23874	1286358 ±124415

[†]Values are given as peak area ± SE of the metabolite as determined by HPLC with fluorescence detection. Six mice were analyzed in duplicate for each of the four groups. ET= ergotamine, M1 = metabolite 1, M2 = metabolite 2 and so forth. $_i$ = epimer of that metabolite.

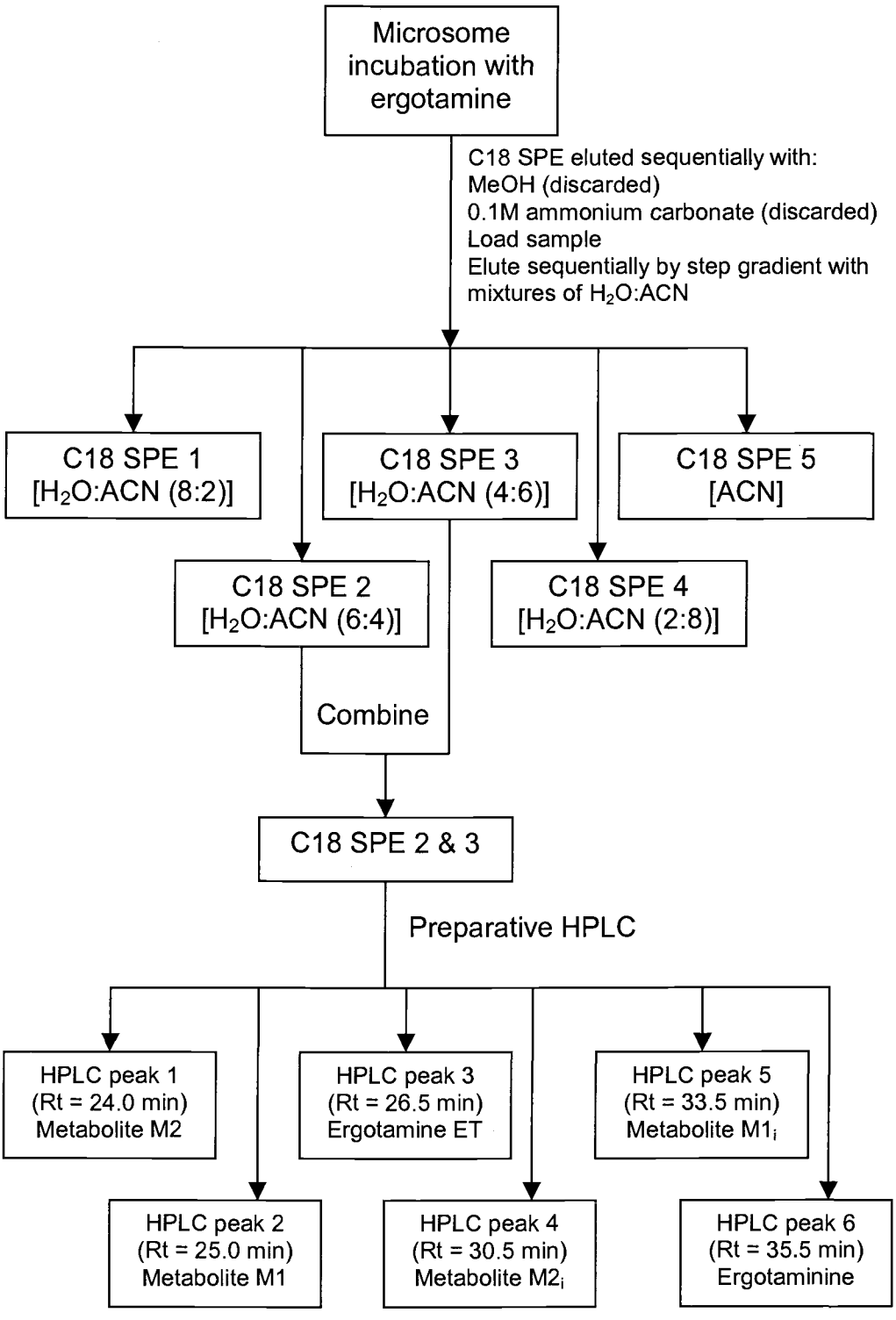


Figure 4.5 Flow diagram for purification of ergotamine P450 microsome mediated transformation products.

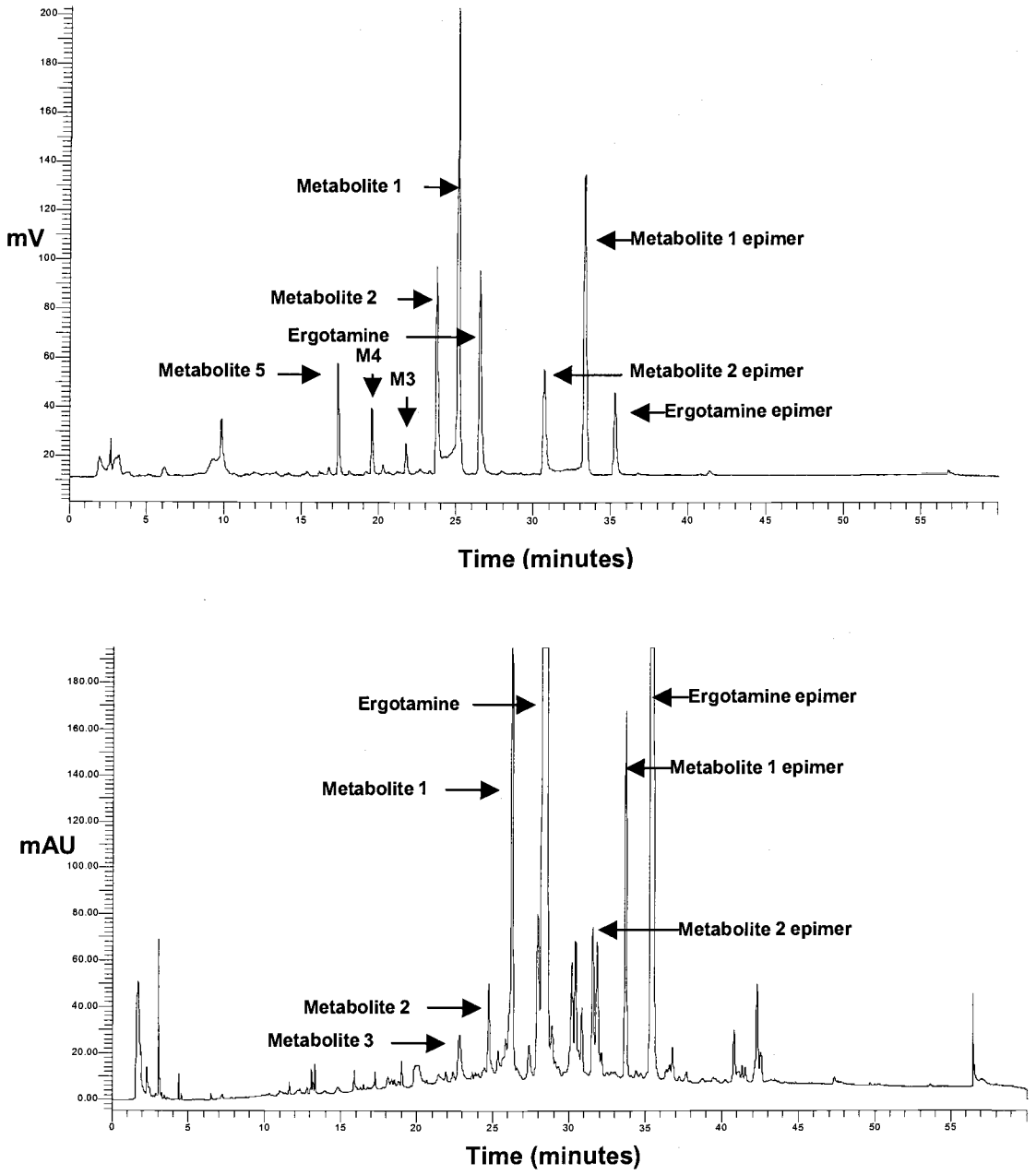


Figure 4.6 Chromatogram of *in vitro* ergotamine incubation with mouse liver microsomes detected by fluorescence (top) and UV (bottom) detection. M4 = metabolite 4, M5 = metabolite 5.

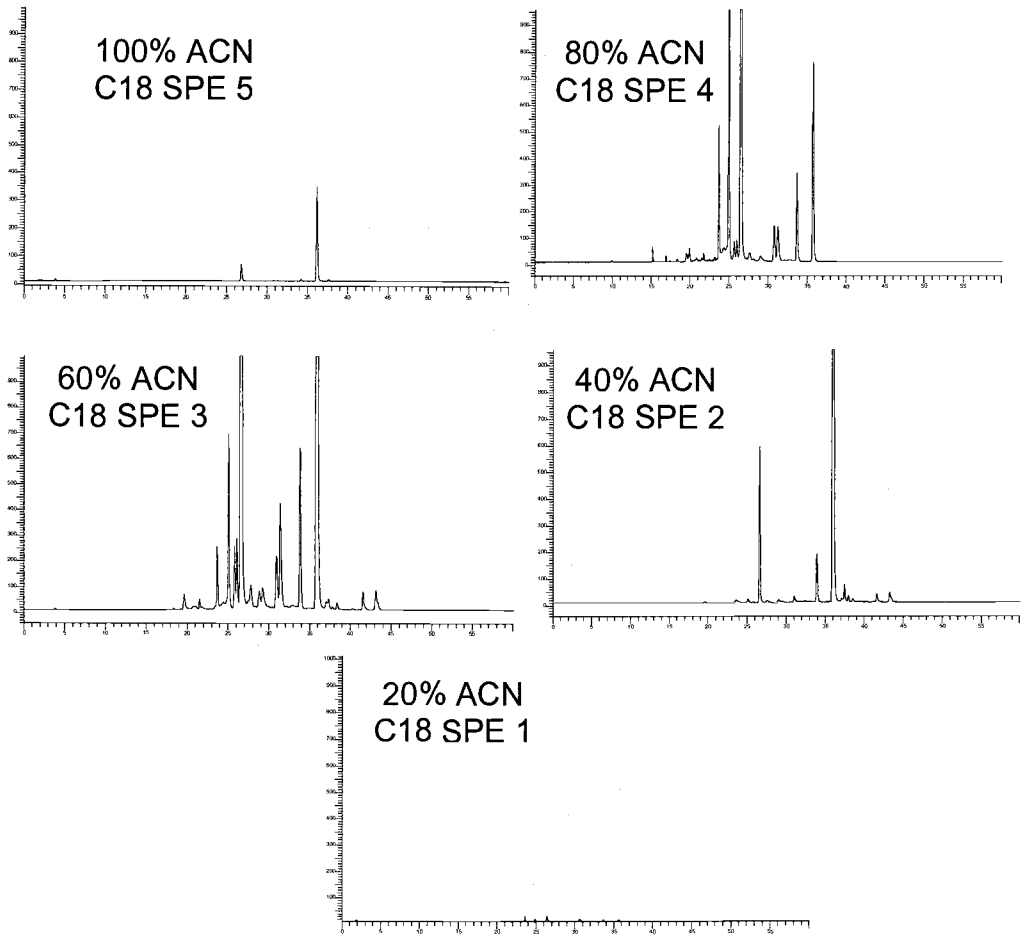


Figure 4.7 HPLC chromatograms of collected C18 SPE fractions as detected by fluorescence (y axis scale = 1000 mAU, x axis scale = 60 minutes for all chromatograms).

separately, dried down under N_2 gas and reconstituted in methanol. The fractions were checked for purity by UV and fluorescence. Chromatograms of these six fractions by fluorescence detection are shown in the top panel of Figure 4.8A-F.

III. LC/MS analysis of ergotamine and its metabolites

Collected HPLC fractions were analyzed by (+) ESI LC/MS. The mass spectra of ergotamine, ergotamine epimer, metabolite 1, metabolite 1 epimer, metabolite 2 and metabolite 2 epimer collected during preparative HPLC are

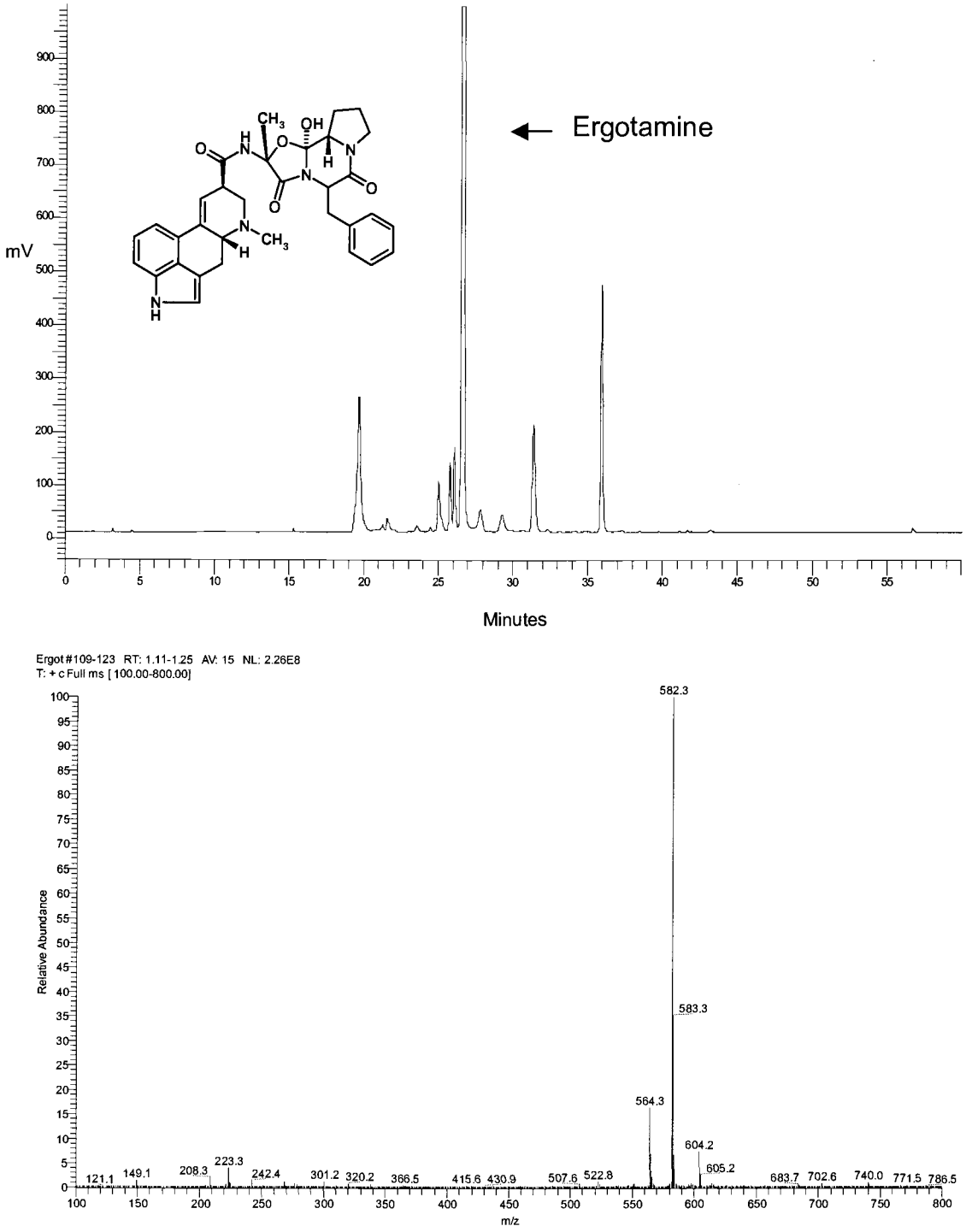
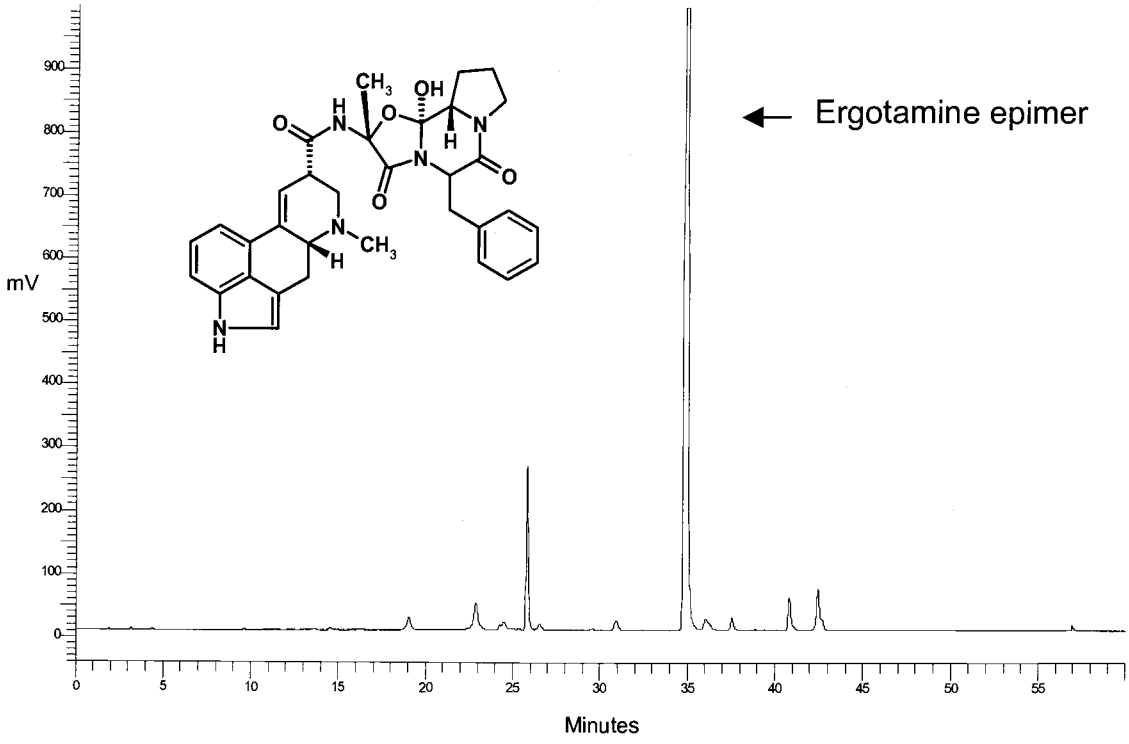


Figure 4.8A Ergotamine fraction detected by fluorescence on HPLC (top) and mass spectrometry (bottom).



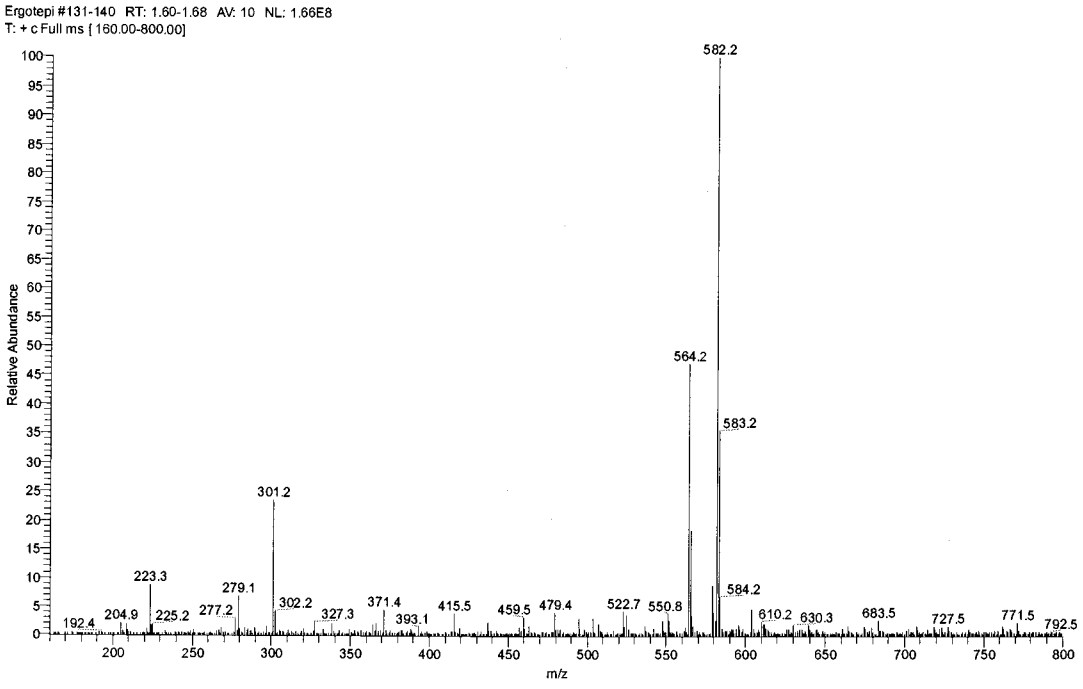
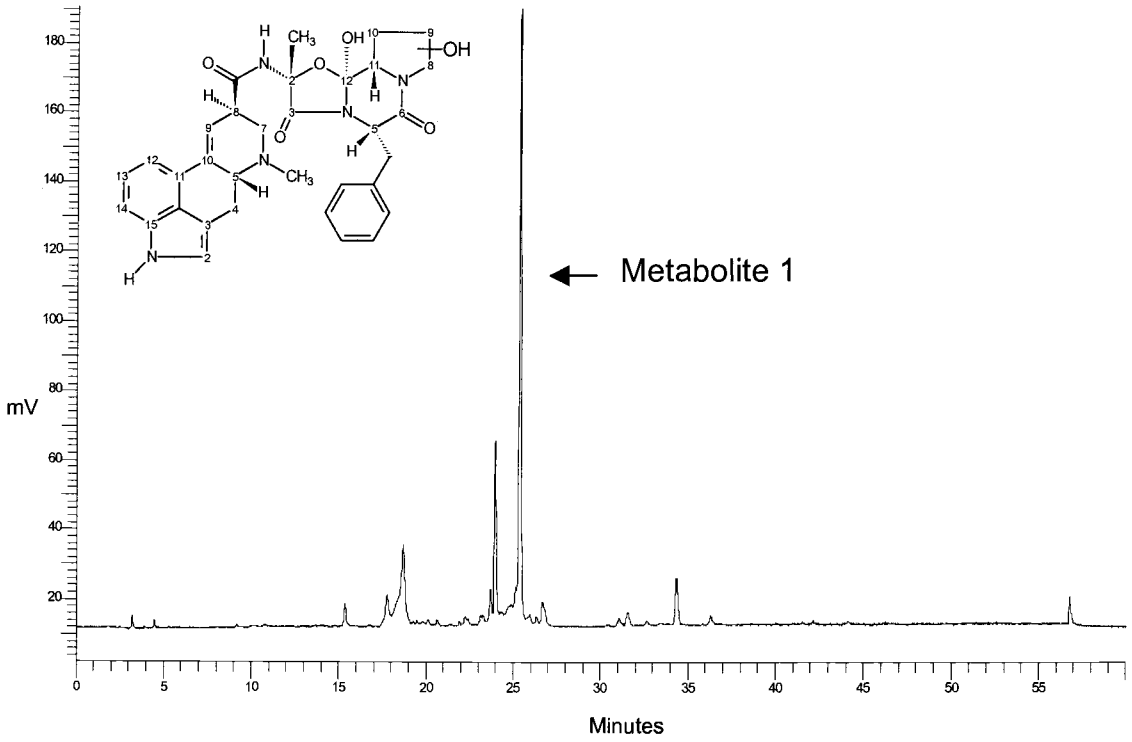


Figure 4.8B Ergotamine epimer fraction detected by fluorescence on HPLC (top) and mass spectrometry (bottom).



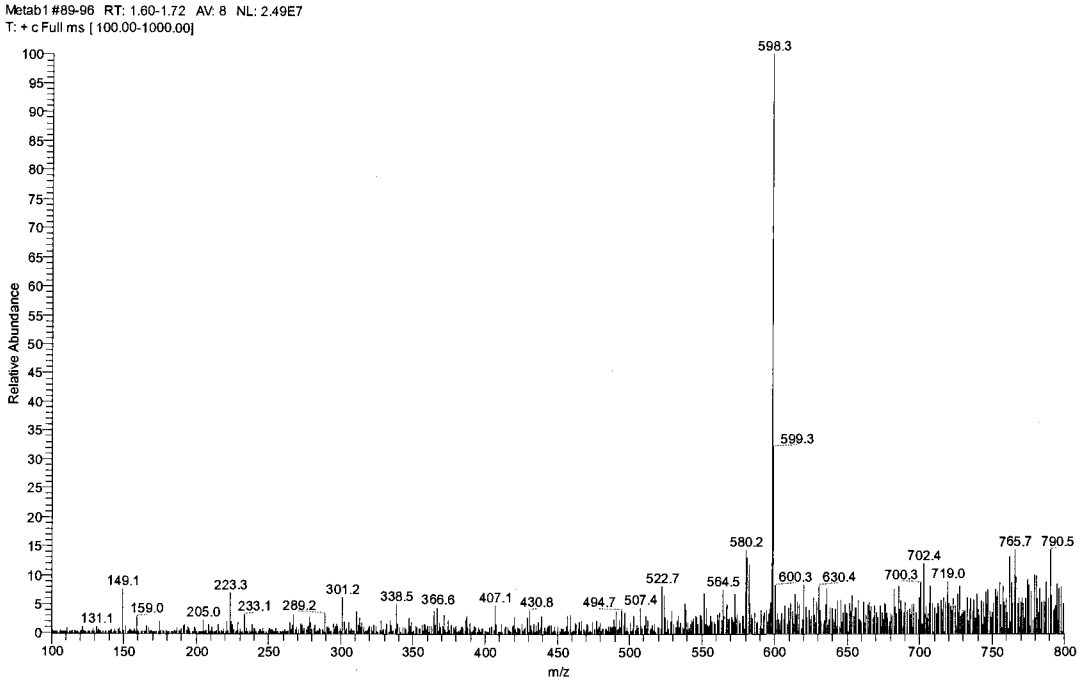


Figure 4.8C Metabolite 1 fraction detected by fluorescence on HPLC (top) and mass spectrometry (bottom).

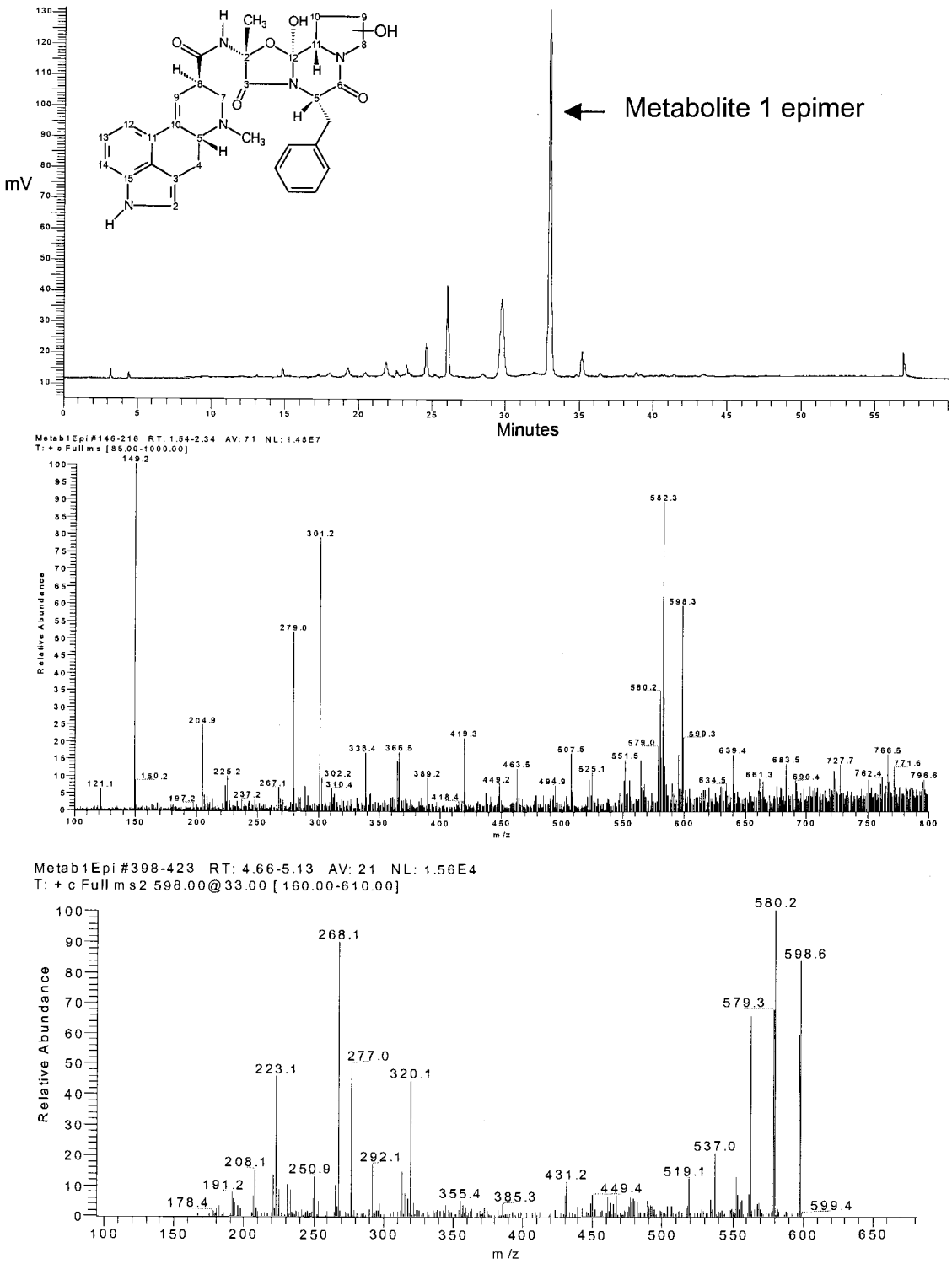


Figure 4.8D Metabolite 1 epimer fraction detected by fluorescence on HPLC (top) and mass spectrometry (middle). MS/MS daughter ion spectra of metabolite 2 at m/z 598 (bottom).

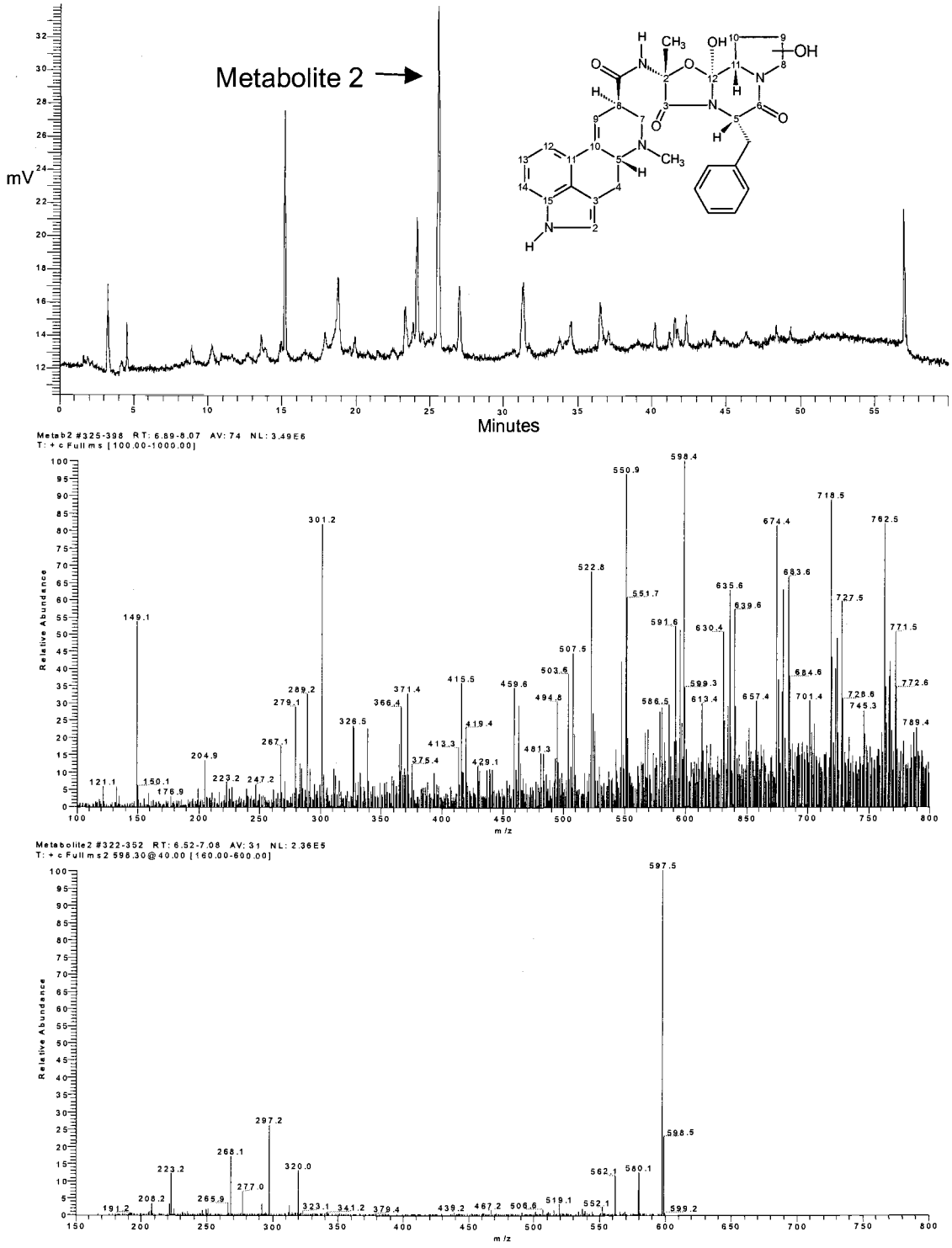


Figure 4.8E Metabolite 2 fraction detected by fluorescence on HPLC (top) and mass spectrometry (middle). MS/MS daughter ion spectra of metabolite 2 at m/z 598 (bottom).

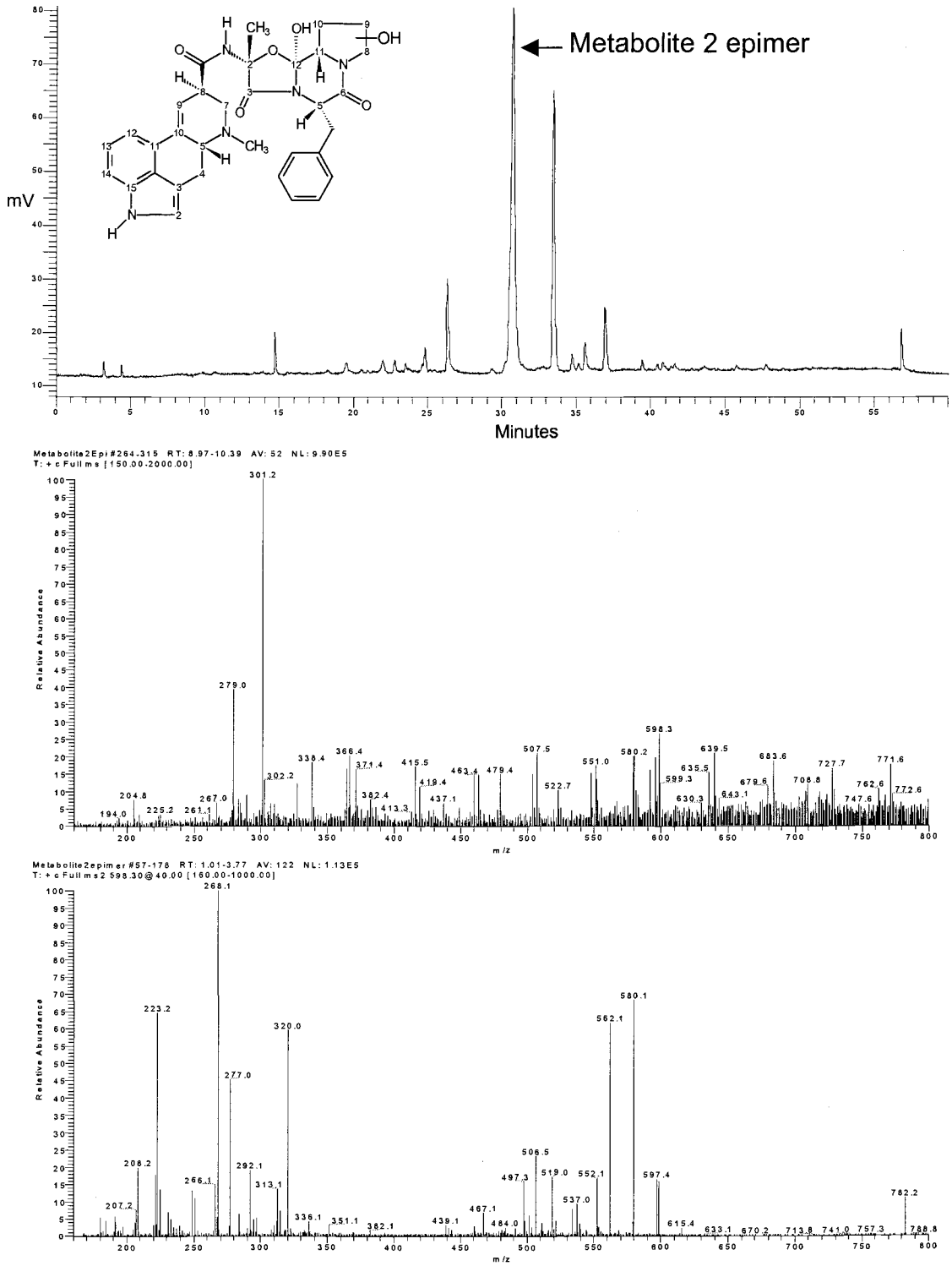


Figure 4.8F Metabolite 2 epimer fraction detected by fluorescence on HPLC (top) and mass spectrometry (middle). MS/MS daughter ion spectra of metabolite 2 epimer at m/z 598 (bottom).

presented in Figures 4.8A-F. Ergotamine and ergotamine epimer fractions produced strong spectra, with a pseudomolecular ion $[M + H]^+$ of 582 which is consistent with ergotamine (Figure 4.8A and 4.8B). These two spectra also had an ion with a m/z = 564 which suggests a loss of water from the molecule. A mass of 598 is consistent for a molecule of hydroxylated ergotamine and was present in metabolite 1 (Figure 4.8C) and metabolite 2 (Figure 4.8E) and the epimer of metabolites 1 (Figure 4.8D) and 2 (Figure 4.8F) in addition to a m/z of 582. A m/z = 223 was evident in all spectra which correlates to the lysergic acid ring system on the molecule.

The spectra from metabolite 2, metabolite 2 epimer and metabolite 1 epimer were not as strong as the other spectra probably due to impurities in the fraction. The m/z of 598 present in all spectra suggests that these fractions are consistent with hydroxylation of the ergotamine molecule. To further study the identity of the metabolite 1 epimer, metabolite 2 and metabolite 2 epimer fraction, a MS/MS scan was performed for ions with a m/z of 598 only (Figure 4.8D-F). The daughter ion spectra produced signals for m/z of 598, 580, 320, 268, 251, 223 and 208. A m/z of 598 is consistent with hydroxylated ergotamine. A m/z of 580 is consistent with the loss of water from hydroxylated ergotamine. A m/z of 320 is consistent with cleavage of the peptide ring system (in amide and at ether) with transfer of a proton. A m/z of 268 is consistent with the lysergic acid ring system + H^+. A m/z of 251 is consistent with a molecule of m/z 268 without a NH_3 group. A m/z of 223 is consistent with the lysergic ring system without the acidic carboxyl group at C8 of the lysergic ring system. A m/z of 208 is consistent with a molecule of m/z 223 without the CH_3 group bonded to the N at position 7 in the ring system. The presence of intact lysergic acid ring system fragments further suggests that the hydroxylation takes place on the peptide ring system of the molecule for this metabolite. The large m/z peak of 301 which is present in some of the metabolite spectra is not present in the daughter ion spectra, suggesting that m/z 301 is an impurity in the mixture.

Discussion

One of the objectives of this study was to determine whether the genetic divergence established in previous generations of the endophyte-resistant mouse line had persisted over ten generations of relaxed selection. Weight gain criteria established in previous studies on these mouse lines was used to determine if an E+ diet adversely affected weight gain between lines or genders of mice. Differences in the average daily gain of mice were significant between both gender and lines during the first two weeks of the experiment with E- feed (Table 4.1). Surprisingly, susceptible mice appeared to gain more weight per day during the first two weeks post-weaning than resistant mice. This is in opposition to other studies which found suppressed weight gain in susceptible mice and used this as a selective tool in continuing these divergent lines (Wagner et al. 2000, Hohenboken and Blodgett 1997). There were larger differences in ADG when genders of the same line were compared as males exhibited a larger ADG than females. This is observed in normal, unexposed ICR mice where males are generally ~10 grams heavier than females during 8-10 weeks of age. Therefore, this gender difference cannot be attributed solely to the selection for divergent lines in response to endophyte-infected feed in previous generations.

Differences in the average daily gain between resistant and susceptible lines when challenged with endophyte-infected feed found in previous studies (Wagner et al. 2000, Hohenboken and Blodgett 1997) was no longer evident in the mice used in this experiment (Tables 4.1 and 4.2). Resistant mice were actually more adversely affected than susceptible mice, although only slightly so. When maintained on an endophyte-free diet, the ADG trend reversed between resistant and susceptible lines observed during the first two weeks. Resistant mice showed a 0.03 g/day larger ADG than susceptible mice. Differences were maintained between males and females. When compared

between diets, resistant and susceptible lines both exhibited a larger weight gain on the E- diet than on the E+ diet.

Differences of susceptible and resistant lines of mice were further investigated by looking at ergotamine transformation by liver microsomes. Lack of affordable, large quantities of ergovaline presents a roadblock in toxicity studies of this compound, so other ergot alkaloids with a similar chemistry are often used in place of ergovaline for research (Garner et al. 1993). Thus, ergotamine was the ergot alkaloid chosen for the *in vitro* metabolism studies conducted here since it has a chemical structure similar to ergovaline and is available commercially.

Early work on bromocryptine metabolism in rat liver preparations identified two hydroxylated metabolite isomers at C-8 of the peptide moiety, hydrolytic cleavage of the amide bridge to yield 2-bromolysergic acid and epoxidation at C-8 to yield dihydroxy derivatives by FD-MS, EI-MS and ^{1}H-NMR (Maurer et al. 1982). Other research using bromocryptine and dexamethasone-induced rat liver microsomes showed formation of four main polar metabolites (Peyronneau et al. 1994). They identified two primary oxidation metabolites in the first 15 minutes of incubation which were then oxidized further into two other metabolites. Mass spectral analysis of the first two compounds showed a *m/z* of 670, corresponding to incorporation of an oxygen atom to the bromocryptine molecule (*m/z* 654). The last two compounds had a *m/z* of 686, suggesting the addition of a second oxygen atom to the structure. They also found a mass fragment with bromine of *m/z* 346, which corresponds to the lysergic part of bromocryptine. This indicates that this part of the molecule remained intact after microsomal oxidation. These researchers also measured the four metabolites using an ELISA with an antibody specific for the bromolysergic part of bromocryptine. Its affinity was high for bromolysergic acid, fourfold lower for bromocryptine and low for the other metabolites. If the lysergic acid part of the molecule had been oxidized, this would have led to low affinity of this metabolite for this antibody which was not the case.

Together, this suggested that the first two metabolites were hydroxylated at the tripepide moiety and that the second two metabolites were further hydroxylated at the tripeptide part of the molecule. In addition, they suggest that the first two metabolites are stereoisomers of 8'-hydroxybromocryptine and that the second two are 8',9'-dihydroxybromocryptine stereoisomers, based on previous research (Maurer et al. 1983, Maurer et al. 1982).

The current study results were consistent with hydroxylation of ergotamine (Figures 4.8C-F). Mass spectroscopy results gave pseudomolecular ions of m/z 582 for the ergotamine and ergotamine epimer peaks that were consistent with the mass of ergotamine (Figure 4.8A-B). It appears that the oxidative products formed as M1, $M1_i$, M2 and $M2_i$ in the *in vitro* incubations are hydroxylations of ergotamine. The presence of the pseudomolecular ion m/z 564 corresponds to a loss of water from the molecule, while the m/z 598 present in both metabolites 1 and 2 and their epimers corresponds to hydroxylation of the molecule. The m/z ion 223 corresponds to lysergic acid and, although weak, was present in all of the metabolites. The daughter ion spectra of m/z 598 from metabolite 1, metabolite 2 and metabolite 2 epimer showed multiple fragments that were consistent with an intact lysergic acid ring system. Based on this data and information available in the literature on identification of ergot alkaloid metabolites by MS and NMR (Shelby et al. 1997, Peyronneau et al. 1994, Maurer et al. 1983, Maurer et al. 1982), it is likely that the oxidative activity taking place in the mouse microsomal preparations is located on the peptide side of the molecule and not in the lysergic ring system since this portion of the molecule appears to remain intact in all spectra.

Ergotamine and other ergot alkaloids are extremely susceptible to photolytic and air oxidation, hydration and isomer formation at C-8 of the lysergic acid ring system (Moubarak et al. 2003) (Figure 4.1). Both enantiomers are present in the incubation mixture since the molecule is in active equilibrium between the two isomers via an enolate intermediate. When studying bromocryptine, Maurer et al. 1982 found that none of the metabolites

showed oxidation of the bromolysergic half of the molecule and that C-8 of the peptide portion of the molecule plays a central role in the hydroxylation of the molecule. Once hydroxylated, this carbon becomes a chiral center which results in two possible isomers that can be separated by HPLC. A second hydroxylation at C-9 of the peptide ring would create a second chiral center, creating two additional isomers which can be separated by HPLC. Thus, two chiral centers are present on the molecule and since the number of isomers is equal to 2^n where n equals the number of chiral centers, there are four possible molecules that can be separated and detected by HPLC. These would appear as pairs in an HPLC chromatogram in that where ergotamine and its isomer elute, the metabolite peaks produced as a result of those two molecules would appear in similar positions relative to their parent molecule. Such is the case in the metabolism of ergotamine and its isomer in mouse liver microsomes (Figures 4.6). Previous to ergotamine and its isomer, metabolites 1 and then 2 appear in increasing distance from the parent molecule giving the four peaks predicted from the two chiral centers.

Bromocryptine metabolism was studied further by following the biotransformation of radiolabeled bromocryptine *in vivo* in mice, rats, dogs and rhesus monkeys and *in vitro* in humans (Maurer et al. 1983). Bile was confirmed as the main route of elimination for bromocryptine and its metabolites as >90% of the oral or intravenous dose was excreted in the feces. They also found similar metabolite profiles in all animals studied, leading them to conclude that bromocryptine metabolism occurs through the same principal pathways in all investigated species. This lends credence to the theory that since species like mice are easier and less costly to maintain in laboratory experiments, they can serve as a model to study ergot alkaloid metabolism in depth, with application to livestock and humans.

In conducting these studies, we wanted to determine if the differences in susceptibility to toxicity seen in the two lines of mice were due to differences in the oxidative enzymes in liver microsomes. If the alkaloids differentially induce

activity of oxidizing enzymes in the lines of mice soon after treatment with E+ feed, then we would observe differences in the profile of ergotamine and its metabolites between both lines of mice. On the other hand, if oxidative enzymes do not play a role in the differential susceptibility to endophyte toxicity in these mouse lines, then similar metabolic profiles would be observed. When all mice were consuming the E- diet, the most significant effect seen within lines or gender was the increased production of most metabolites in female versus male mice. This resulted in a lower concentration of ergotamine and its epimer in females (Table 4.3). This is not surprising due to the fact that many compounds undergo differential metabolism between genders of the same species due to expression of different cytochrome P450 isozymes (Williams et al. 1989, Mattocks 1986). Cytochrome P450s and other microsomal enzymes are responsible for the metabolism of endogenous compounds including hormones and other molecules that vary greatly between genders or are gender-specific. This, combined with genetic polymorphisms for specific P450 enzymes and/or previous exposure to compounds that can induce or inhibit P450s, can lead to large differences in concentration and activity of P450 isozymes between individuals (Klaassen 1996). Cytochrome P450 concentration or specific isozyme activity and concentration were not measured in this study, so differences in the metabolism of ergotamine due to differential P450 expression cannot be quantified. However, the same concentration of microsomal protein was used in all *in vitro* incubations in this study. This is a standard practice when conducting P450-mediated metabolism studies with liver microsomes. If all microsomal preps are made in the same way, then it is likely that a given concentration of protein contains relatively similar amounts of total P450 in animals tested under controlled experimental conditions.

Nonetheless, it would be of interest in future studies to quantify these isozymes as they are known to play a role in the oxidative metabolism of many ergot alkaloids (Moubarak et al. 2003, Althaus et al. 2000, Moubarak and

Rosenkrans 2000, Peyronneau et al. 1994, Gautier et al. 1993, Ball et al. 1992, Moochhala et al. 1989, Niwaguchi and Inoue 1975). The CYP3A family has been cited as the main P450 enzyme responsible for the metabolism of a variety of ergot alkaloids (Moubarak et al. 2003, Althaus et al. 2000, Moubarak and Rosenkrans 2000, Peyronneau et al. 1994, Ball et al. 1992), with mono- and dihydroxylation and *N*-dealkylation as the main oxidative processes carried out be these enzymes. Recent experiments performed in bovine and dexamethasone-induced rat microsomes with ergotamine and ergonovine showed a similar metabolite profile as that seen in this study (Moubarak et al. 2003, Moubarak and Rosenkrans 2000). This suggests that metabolism of ergotamine in the divergent mouse line microsomes is carried out by CYP3A as well, which would be of interest to investigate in future studies.

After the feeding experiment with endophyte-infected seed, the trends in ergotamine metabolism became less distinct (Tables 4.4 and 4.5). Difference in the formation of ergotamine and its epimer between male and female mice was maintained after initiation of the feeding experiment. However, metabolite 1 and its epimer, suggested to be hydroxylated metabolites from the LC-MS data, showed no significant differences between lines or gender. There was a weak relationship for the line*sex variable for metabolite 2 and its epimer (also hydroxylated metabolites), which suggests that effects from a mouse's line and gender interact in determining the relative formation of metabolite 2 and its epimer. This interaction term makes it difficult to separate out differences in ergotamine metabolism into general line and gender effects.

For formation of metabolites 3-5, differences between gender were maintained and were significant for the line*sex interaction term as well. Metabolite 3 formation also differed between resistant and susceptible lines, with resistant mice forming more of metabolite 3 than susceptible mice. Metabolite 4 was an exception in the feeding experiment in that it showed marginal to significant differences in all but the line*feed interaction. Resistant males formed more of this metabolite than susceptible males but the trend

reversed in females, with susceptible females forming more relative to resistant females. Based on previous research (Peyronneau et al. 1994, Maurer et al. 1982), it is suggested that these are dihydroxylated metabolites of ergotamine. They are formed from 8'-hydroxy isomers which appear first in incubations and then decrease as the dihydroxylated metabolites increase in concentration (Moubarak and Rosenkrans 2000).

The loss of genetic divergence in response to endophyte toxicity as measured by weight gain and ergotamine metabolism in this study has been seen to some extent in previous work done with these lines of mice. Wagner et al. (2000) reported that as time progressed, differences between the lines in response to the toxins from endophyte-infected feed moderated as susceptible mice became somewhat less susceptible as they aged and weaned a similar number of pups as the resistant mice. This lead to the conclusion that resistance to fescue toxicosis is expressed early in the reproductive stage of an individual and is not magnified as an animal ages. Moreover, susceptibility may moderate over time.

The finding of gender differences in metabolism of ergotamine to many of the identified metabolites has not been shown before in these lines of mice or other species used to study ergot alkaloid metabolism. This adds a potential source of variation not explored previously and would be of value to investigate further. Further characterization of the Phase I and II enzymes and ergot alkaloid metabolic products in these lines would provide a more complete analysis of metabolic differences or similarities between genders and between the lines.

Analysis of metabolites 3-5 by LC-MS was not performed due to the small quantity of compound produced relative to other metabolites and the cost in conducting these assays. It would be of interest to identify these metabolites, as it would lend a more complete picture of hepatic metabolism of ergotamine in the two lines of mice. Of particular interest are metabolites 3 and 4 which exhibited significant line and gender differences. It is postulated from previous

studies (Moubarak and Rosenkrans 2000, Peyronneau et al. 1994, Maurer et al. 1982) that these could be 8', 9'-dihydroxylated stereoisomers of ergotamine. In addition, since it has been suggested that ergovaline is the alkaloid responsible for fescue toxicity, it would be of interest to study hepatic metabolism of this alkaloid in sheep and cattle who exhibit different susceptibilities to fescue toxicity.

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Chapter 5

SUMMARY

The work presented herein centered around characterizing the mechanisms of hepatic toxicity from plant alkaloids in livestock. Interspecies variability exists in the toxicity of pyrrolizidine alkaloids such that cattle are susceptible and sheep are resistant. The mechanism of resistance to toxicity has been attributed to two factors: 1) increased efficiency of ruminal microbes in PA detoxification and/or 2) production of less toxic metabolites in hepatic metabolism of these compounds. Differences in ruminal metabolism between sheep and cattle have been demonstrated as a mechanism for detoxification of PAs in sheep, a contributing factor in their resistance to toxicity. Thus, the *in vitro* hepatic metabolism of the PA senecionine was studied in sheep and cattle to determine the role of hepatic bioactivation and detoxification in PA toxicity.

Previous research has shown that production of the toxic 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) influenced an animal's susceptibility to PA toxicity. However, no difference was seen in the concentration of DHP formed between sheep and cattle in this study. Sheep did exhibit a higher rate of formation of the relatively non-toxic senecionine *N*-oxide and higher concentrations of the enzymes thought to be responsible for their formation. Sheep and cattle exhibited similarities in the influence of cytochrome P450s on DHP formation and flavin-containing monooxygenases on senecionine *N*-oxide formation. Both species also exhibited a decrease in DHP formation when glutathione was added to incubations, suggesting that glutathione conjugation plays an important role in detoxification of PAs in both species.

A concurrent *in vivo* study involving sheep and cattle that looks at the metabolism of PAs by the microbial population of the rumen as well as liver

samples profiling the presence and activity of Phase I and Phase II enzymes and their metabolism of PAs would allow a side-by-side comparison of both theories of susceptibility to PA toxicity. Also relevant to an *in vivo* study would be measurement of PA, *N*-oxide and DHP both in the rumen and the liver. This would help elucidate the fate of ingested PAs in sheep and cattle. In addition, investigation of the gastrointestinal metabolism of PAs by Phase I and Phase II enzymes in the small intestine in sheep and cattle would provide more information on metabolism of PAs before they undergo metabolism in the liver. The potential toxicity of *N*-oxides would also be interesting to investigate as most plants contain a large proportion of PAs in this form. Direct incubation in *in vitro* assays similar to those performed in this study would yield information on their ability to be metabolized back to the parent PA for passage through the three metabolic pathways discussed.

Detection of ergot alkaloids produced from endophyte-infected tall fescue plants by an alternative assay was investigated. It was determined that the ELISA assay was semiquantitative due to the daily and within-run variability but could be used as a tool to detect the presence of ergot alkaloids with a structure similar to lysergic acid. In addition, the ELISA assay exhibited similar variation for clarified rumen fluid standards compared to standards in a grass-tissue based solution. Thus, this assay is adaptable to other matrices.

Differences in weight gain and metabolism of the ergot alkaloid ergotamine were investigated in mice that had been previously selected for susceptibility or resistance to endophyte toxicity. No differences were seen in weight gain between mouse groups. Ergotamine metabolism showed a difference between genders in mice on an endophyte-free diet. Once challenged with an endophyte-infected diet, both genders again exhibited a difference in formation of some of the biotransformation products. A difference was also exhibited between animals on endophyte-infected and endophyte-free diets for some of the more polar metabolites. Analysis of the metabolites by LC-MS

confirmed the identity of ergotamine and its isomer in addition to four hydroxylated ergotamine transformation products.

Basic research is need to determine which of the alkaloids is responsible for fescue toxicosis in livestock. Investigation on the metabolism and toxicity of specific ergot alkaloids is needed to better characterize the mechanism of fescue toxicity. Studies following the fate of ergot alkaloids in both the rumen and liver would provide necessary information on the metabolism of ergot alkaloids to other non-toxic or toxic moieties. To that end, the identification of the other metabolites formed from ergotamine metabolism in mouse liver microsomes should be included in future studies. The role of cytochrome P450s and flavin-containing monooxygenases in metabolism of ergot alkaloids in livestock is just beginning to be investigated. Studies on how these enzymes are involved in ergot alkaloid metabolism have application to human toxicity as well in that many ergot alkaloids are used to treat human diseases. Lastly, development of a rapid, inexpensive assay to detect ergot alkaloids would be of great value to grass and livestock producers due to increasing regulations on the export of endophyte-infected hay.

Research on the fate of ergot alkaloids once ingested from endophyte-infected grass are currently being conducted at this laboratory in steers. Recently, Dr. Craig's laboratory conducted an *in vivo* experiment which involves a 4 X 4 Latin square design of four fistulated 4 year old steers each receiving one of four endophyte-infected fescue diets over a 21 day feeding period. These four fescue diets had a concentration of <40, 220, 350 or 500 ppb ergovaline. Each steer received each treatment during one of the four feeding periods, so all animals were exposed to all doses. At the end of each two week period, blood, rumen, urine and fecal samples were taken and stored for ergovaline, lysergic acid and loline alkaloid analysis to determine the fate of ergot alkaloids from tall fescue as they are ingested. All steers were weighed to determine if the various levels of E+ fescue had an effect on weight maintenance.

The results of this study will help determine how E+ fescue affects weight maintenance in steers. Other work currently being done in this lab includes *in vitro* experiments with sheep and cattle rumen fluid inoculated with various ergot alkaloids. The fate of these alkaloids over 24 and 48 hours is being studied and quantified by HPLC.

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