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**Pharmacokinetic study of slaframine in lactating goats and
transfer into milk**

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Pharmacokinetic study of slaframine in lactating goats
and transfer into milk

by

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ABSTRACT

Slaframine is an alkaloidal mycotoxin produced by the fungus *Rhizoctonia leguminicola*. This mycotoxin is responsible for "Blackpatch" disease on clover and other legumes and "Slobber" disease in livestock. Slaframine causes salivation, lacrimation, urination, and defecation in intoxicated animals.

The goal of this research was to do a preliminary pharmacokinetic study on slaframine in lactating goats and determine if slaframine was transferred into milk. Slaframine was produced to dose five lactating Saanen goats at a level of 0.05 mg/kg. The slaframine was delivered as an IV bolus dose. Blood, milk, and saliva samples were collected at timed intervals.

All samples were analyzed by a newly developed HPLC procedure for slaframine. This procedure used fluorescamine derivatized slaframine to enhance detection 10-fold over previous GC technology. The slaframine derivative was found to be stable for several weeks. The mean recoveries of slaframine from plasma, milk, and saliva were 95%, 91%, and 82%, respectively.

The pharmacokinetic study indicated a two compartment model. The half-life for the elimination phase derived for the sample-mean of all five goats was 1.28 hours. The volume of distribution indicated that slaframine was highly protein-bound in goat blood. The mean clearance value of slaframine was 2.6 ml/kg/h, indicating a rapid elimination of slaframine from the body.

Due to the unpredictably long half-life of slaframine in the milk, sampling duration

was insufficient in all but one goat to study the pharmacokinetics of slaframine in milk. This data would best describe the pharmacokinetic behavior as a two- or three-compartment model. As a two-compartment model, the elimination phase of slaframine in milk was 23 hours. To describe the data as a three-compartment model, (with or without a deep compartment) lower levels of slaframine would need to be detected in plasma. Higher levels of slaframine were seen in milk than in plasma, which correlates well with the pH partition theory. A milk/plasma ratio of 20:1 was obtained using experimental pH values.

As a result of slaframine transfer into milk, a potential hazard exists for nursing goats and consumers of goat milk and its by-products. Further studies are necessary for making risk assessments for young animals with the occurrence of slaframine in milk.

I. LITERATURE REVIEW

Slaframine is an alkaloidal mycotoxin produced by the fungus *Rhizoctonia leguminicola*. This mycotoxin is believed to be responsible for "Blackpatch" disease on clover and other legumes and "Slobber" disease in livestock. "Blackpatch" disease was aptly named for the dark spots of fungal growth seen on clover. "Slobber" disease was given its name because of the excessive salivation that occurred in animals following the consumption of fungal contaminated feed.

A. Reported History

"Blackpatch" was reported on red and white clover in Kentucky as early as 1933 (1,2). In 1937, Smith (2) reported the presence of "Blackpatch" in Wisconsin. In 1947, Gough and Elliott (3) gave the name *Rhizoctonia leguminicola* to the fungus that causes "Blackpatch" disease. In 1950, the disease was reported in both Georgia and West Virginia. In Georgia, "Blackpatch" was reported on soybeans and other legumes in addition to clover. In 1951, Leach and Elliott (2) reported that the incidence of the disease varied from year to year: dependent upon the use of infected seed.

Livestock displaying the symptoms of "Slobber" disease were observed in Missouri, Illinois, Indiana, and Ohio prior to 1950 (4). O'Dell (5) documented a slobbering problem in Missouri that continued for several years, from 1947 to 1958. The occurrence of the disease has also been reported in Delaware, Maryland, Pennsylvania,

Iowa, Tennessee, Virginia, and North Carolina (4,5,6,7).

B. Fungal Characteristics

Rhizoctonia leguminicola is a pathogen that kills its host plant as it spreads (8). Concentric zonation occurs on the leaf in the early period of infection. The fungus then moves rapidly through the plant tissue, and aerial mycelia infect new leaf surfaces. Irregular lesions are formed by the overlap of necrotic areas. The fungus can overwinter on red clover and has been known to last for up to two years in infected seeds (4).

The fungus does not produce spores and for this reason was not named or classified until 1947. The main reasons the "Blackpatch" fungus is in the genus *Rhizoctonia* are: 1) no sporulation, 2) production of sclerotia, and 3) constriction of hyphae at branch points. The sclerotia of *Rhizoctonia leguminicola* are either round or irregular in shape with rough glossy black surfaces and may not mix together to form clumps or irregular masses. Sclerotia have not been observed in nature but only in the cultured fungus when a nitrogen source is present (2).

In pure culture, *Rhizoctonia leguminicola* initially appears dark green in color and as it gets older turns to brown and then black (2). High humidity is needed for aerial mycelia growth. The pathogen, *Rhizoctonia leguminicola*, appears to be very stable in pure culture, and, with annual transfer of the mold, test cultures have continued to produce positive tests in animals for 13 years (4,5). The dried mycelia can be viable up to 6 months (2). All isolates of the fungus are known to produce the toxin (4,5).

"Blackpatch" usually develops during periods of wet weather, high humidity, and mild temperatures between 25 - 29 °C (4,5). The disease symptoms of the plant are obvious but farmers usually associate these with the ripening of the clover.

In addition to red and white clover, other legumes have been infected naturally by the fungus *Rhizoctonia leguminicola*. These include soybean, kudzu, cowpea, blue lupine, alsike clover, alfalfa, Korean lespedeza, and black medic. The disease usually infests other legumes only when they are grown alongside red clover. Ladino clover, crimson clover, white sweet clover, yellow sweet clover, sour clover, and bush beans have been infected using artificial inoculation (4). Regardless of whether infection is natural or induced, no resistant varieties of clover have been found (4,5).

Most reported cases of "Slobber" disease result from red clover hay. In addition, reported cases most often result from hay cut in the fall from the stubble of spring seeding or from second cutting hay (9). Perhaps the high moisture content of these cuttings contribute to fungal growth (8).

C. Isolation

In the early 60's several serious outbreaks of "Slobber" disease occurred where cattle, horses, sheep, and goats had consumed forage contaminated with *Rhizoctonia leguminicola*. In 1962 Smalley and Crump at Wisconsin microscopically examined slobber forage and found the hay to be infected with a heavy dark brown mycelia. They identified the fungus pathogen on red clover hay as *Rhizoctonia leguminicola* (4,5,10,11).

When this fungus was produced in pure culture the mycelial mat was found to cause "Slobber" disease (12); linking it directly to "Blackpatch" disease (5).

The Wisconsin group and another in Illinois simultaneously began working on the isolation and purification of the slobber agent produced by *Rhizoctonia leguminicola*. The toxin was found to be contained in the mycelial mat and not in the culture fluid (3,13). The isolation and purification scheme by the Wisconsin group is shown in Figure 1 (11,12) and, by the Illinois group, in Figure 2 (5,13,14).

In Wisconsin, Crump and colleagues grew *Rhizoctonia leguminicola* as a static liquid culture for 30 days on a media consisting of 100 grams of dried red clover and 100 ml of distilled water. Later they used a defined, nine ingredient, antibiotic, test media developed by Gregory et al. (12).

In Illinois, Aust grew the mold in 300 ml Roux bottles for three weeks as a static culture on 20% second cutting red clover in water at 25 °C. The yield per bottle was approximately one milligram. An increase in toxin production was noted when 0.1% casamino acids were added to the culture. If cultures were submerged or shaken no toxin was produced (3). Aust later used Czapek - Dox broth with yeast extract for culture media (8).

Both groups used a guinea pig assay developed by O'Dell et al. (3) to aid in the isolation of the toxic agent. This assay graded guinea pigs on an index of salivation where the following scores were given: 1+ for slight salivation, 2+ for intermediate

Figure 1. Wisconsin group slaframine purification scheme.

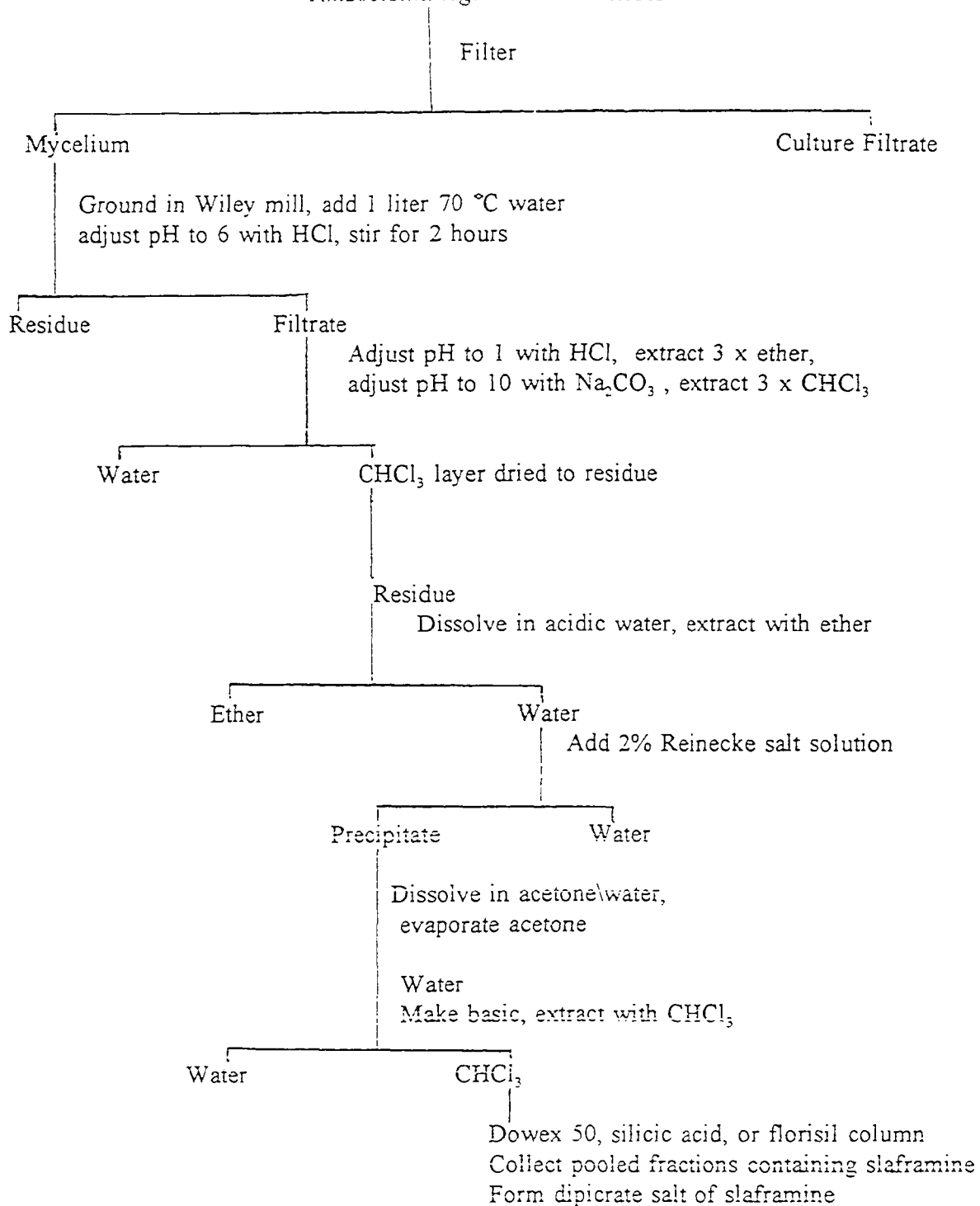
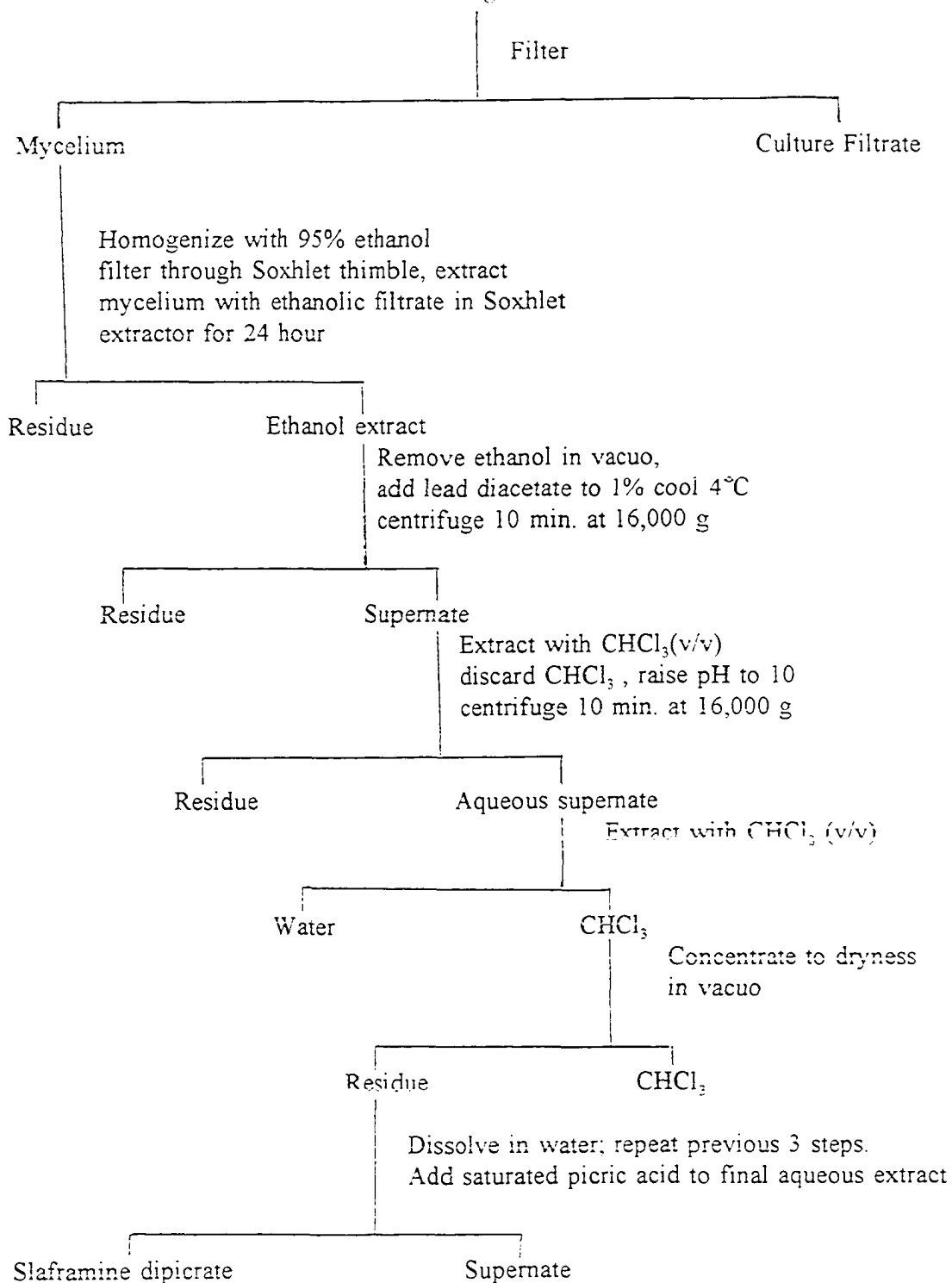
Rhizoctonia leguminicola cultures

Figure 2. Illinois group slaframine purification scheme.

Rhizoctonia leguminicola cultures

salivation, and 3+ for severe salivation (an animal graded 3+ keeps the fur on the throat wet). Solutions of toxin were tested by interperitoneal (IP) injection. Salivation usually occurred within 10 - 15 minutes. If salivation had not occurred within 30 minutes the test was ruled negative (5,13).

D. Purification

The toxic principal has been extracted by either water or ethanol and is soluble in chloroform but not in petroleum ether. The toxin was believed to be an alkaloid because of the pH effects on extraction in aqueous and alcoholic solvents. Slaframine rapidly hydrolyses to deacetyl slaframine at pH levels that are greater than ten. Because deacetyl slaframine has no slobbering effect, care in purification must be taken to avoid this result (8). Upon crude purification, the toxin has given a positive Dragendorff's reaction; strengthening the alkaloid theory (8). Both silica and florisil columns have been used for the purification of slaframine (1,5). Aust (1,5) gave the purified toxin the name slaframine from the old Norse "slafra," which means to slaver.

The purified toxin, as the free base, is a clear reddish colored oil (8,15). The free base has a molecular weight of 198. As a dry film, the free base degrades in the presence of air and is only stable for about two hours (8). However, slaframine is stable in chloroform or methylene chloride for several months without solvent evaporation (8). In addition, while crystallization of slaframine's free base has not been possible (3,15), various salts of slaframine are made for stability (8). Citrate and picrate salt have been

the most stable of these salts in maintaining slaframine for long periods of time (8). The melting point for slaframine dipicrate is 183 - 184 °C (3,11).

E. Chromatography

Analysis techniques for slaframine include both paper and thin layer chromatography (8,12). Aust (11,12,16) used butanol/acetic acid/water (4/1/1, v/v/v) with paper chromatography, giving an R_f of 0.25. He visualized the resulting compound with ninhydrin or Dragendorff's reagents. Byers and Broquist (17) obtained an R_f of 0.12 using paper chromatography with butanol/acetic acid and Dragendorff's visualization.

To show the presence of slaframine in hay extracts, Byers and Broquist (16) used normal phase thin layer chromatography developed with phenol and water; again visualizing with Dragendorff's reagent. Stahr (18) used normal phase thin layer chromatography with chloroform/methanol (80/20, v/v) and visualized the slaframine compound with 5% vanillin in 2% sulfuric acid.

Analysis of slaframine by gas chromatography (GC) has been done using OV 17, OV 1, or SE 30 columns. Hagler (8) used a trimethylsilyl derivative of slaframine for GC analysis. Stahr et al. (19) used an acetate derivative of slaframine for GC analysis.

F. Structure

Preliminary characterization of slaframine using the ninhydrin test or the VanSlyke analysis revealed the presence of a primary amine group (11). Proton nuclear

magnetic resonance (NMR) analysis indicated that a secondary acetate group was present. Further characterization by infrared spectroscopy showed that no double bonds were present. Analysis using cyanogen bromide, which opens the ring structure, demonstrated the presence of a cyclic nitrogen in the ring (20). Investigations of slaframine structure by Aust, Broquist, and Rinehart (20,21,22) used NMR and mass spectrometry to identify the structure as 1-acetoxy-8-aminooctahydroindolizidine. Further proton NMR investigations by Gardiner, Rinehart, Snyder, and Broquist (10,12,23) reassigned the structure to be 1S, 6S, 8aS-1-acetoxy-6a-aminohydroindolizine; changing the amine to the 6 position instead of the 8 position that had been previously assigned.

G. Synthesis

Slaframine has been artificially synthesized by several methods. An early attempt to synthesize slaframine started with 2-bromo-5-nitropyridine and utilized an 11-step process (21). Another method of slaframine synthesis utilized a 20-step process with a Diels-Alder cycloaddition to form the indolizidine ring (24). Two other routes to slaframine synthesis have started with glutamic acid. One used a 13-step process to produce slaframine (25). A later study used glutamic acid and obtained a 6% yield through a 12-step process (26).

The natural synthesis route of slaframine by *Rhizoctonia leguminicola* was discovered using radioactive tracer experiments. Slaframine is formed via the homocitrate aminoadipate pathway, where lysine is formed from acetate, alpha-ketoglutarate, and

glutamate in addition to carbons 2 and 3 of serine (10,12). As shown in Figure 3 (27), the lysine is transformed through a series of reactions to give rise to pipecolate (10,28). The epsilon nitrogen (not the alpha nitrogen) of lysine is incorporated into the pipecolate, which produces the pyridine alkaloids (29,30). Comparisons have demonstrated that using radioactive pipecolate is 1000 times more effective than using radioactive lysine in producing radioactive slaframine (27). A flavin enzyme, saccharopine oxidase, catalyzes the reaction of saccharopine to Δ^1 piperidine-6-carboxylate as one of the steps between lysine and pipecolate. These reactions are not seen in mammals but probably happen in plants (31).

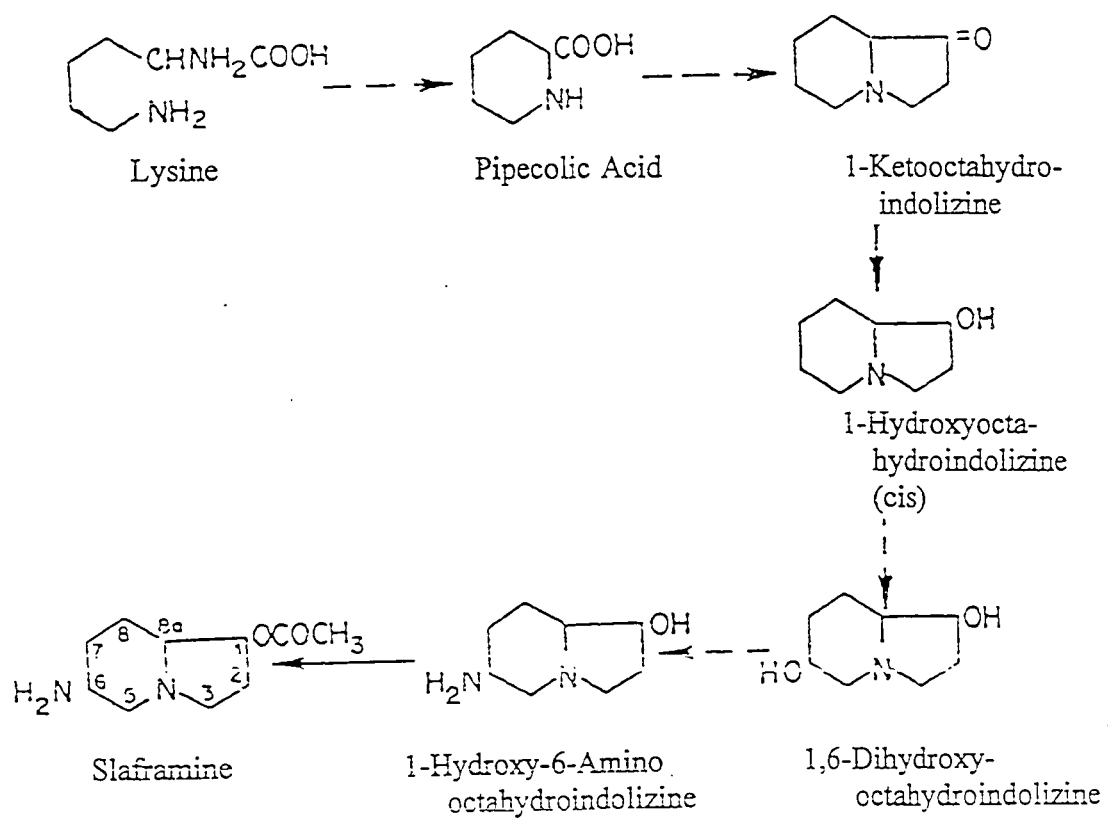
Pipecolate and malonate are further transformed to become slaframine (10,28). The direct precursor to slaframine has been shown to be deacetyl slaframine (27). Among the pyridine alkaloids, slaframine is unique in that the carboxyl carbon derived from lysine remains in the final product (32).

H. Salivation Delay

Early feeding studies using slaframine-infected red clover hay, mycelia, or extracts of cultures from *Rhizoctonia leguminicola* showed a delay time from oral dose to salivation, the most apparent clinical response. The delay time period varied with respect to the type of experimental subjects. Delays were observed ranging from 10-30 minutes in experimental laboratory animals and extending up to several hours with cattle (12).

Early physiological studies used crude extracts of slaframine and, somewhat later,

Figure 3. The biosynthesis of slaframine from lysine by *Rhizoctonia leguminicola*.



purified slaframine. Both resulted in a delay time before a response. This suggested that a bioactivation of slaframine was required for physiological action (3).

Aust (8,10,11) systematically delayed the onset of salivation in rats by clamping off the portal vein and hepatic artery for varying time periods while injecting slaframine into via the vena cava. The more direct the route to the liver the less the time to salivation onset. Slaframine was shown to be most active when given via the portal vein. This suggested that the liver was the major site for metabolizing slaframine and where the active metabolite could be found. This was supported by Froetschel (8), whose experiments with cattle showed that the more indirect the route of slaframine to the liver, the longer the delay for salivation. Slaframine dosed orally results in the longest delay; followed, in order, by subcutaneous (SC), interperitoneal (IP), and intervenous (IV) doses.

The induction of liver enzymes by compounds such as phenobarbital or DDT significantly shortens the delay time preceding the activity of slaframine (5,11). In addition, when such inducers have been used, lower doses of toxin have elicited symptoms from animals (33). In direct contrast, inhibitors of liver endoplasmic reticulum (i.e. SKF 525A, SKF 8742A, DPEA, and Lilly 18947) have all been shown to delay the onset of slaframine activity (5,11,34). These results provide further evidence that the activation of slaframine takes place in the liver; possibly at the endoplasmic reticulum (5,11,34).

The delay of salivation onset has also been correlated with the ability to metabolize drugs. Aust stated that goats are good xenobiotic metabolizers and calves are poor xenobiotic metabolizers (33). As a result, goats display a shorter salivation response

than do calves.

I. Activation of Slaframine

Further experimentation involving the activation of slaframine studied both in vivo and in vitro activation. It was not until the activation could be done in vitro that the identity of the active metabolite was discovered.

The incubation of slaframine with liver microsomes and NADPH stimulated contraction in the guinea pig ileum. Atropine could not reverse this. Also, using 80% carbon monoxide and 20% oxygen, no inhibition of the microsomal reaction that activates slaframine was observed. This result indicated that cytochrome P450 was not involved. An indication that the reaction is enzymatic resulted from the observation that boiled microsomes were not able to activate slaframine. Studies using microsomes suggested an oxidative pathway for the activation of slaframine. As a result, it is believed that a flavoprotein oxidase plus NADPH plus oxygen oxidizes slaframine in the liver (34).

In vitro, flavinmononucleotide (FMN) with light was shown to activate slaframine. This resulted in a reduction of FMN at 455 nm. This knowledge was used to further study the active metabolite of slaframine. It was found that the slaframine activated in vitro could not be extracted by organic solvents but could be isolated by cation exchange chromatography. This resulted in the discovery of the active metabolite, 6-ketoimine slaframine. This structure was confirmed by reducing the active compound of slaframine with deuterated sodium borohydride. After this reaction two deuteriums were present in

the product: one from the ketone resulting in a hydroxyl group and one from the reduction of the double bond in the ring structure (5,10). Similar results have been produced using tritiated sodium borohydride.

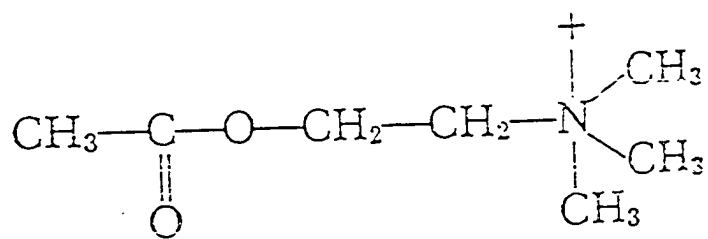
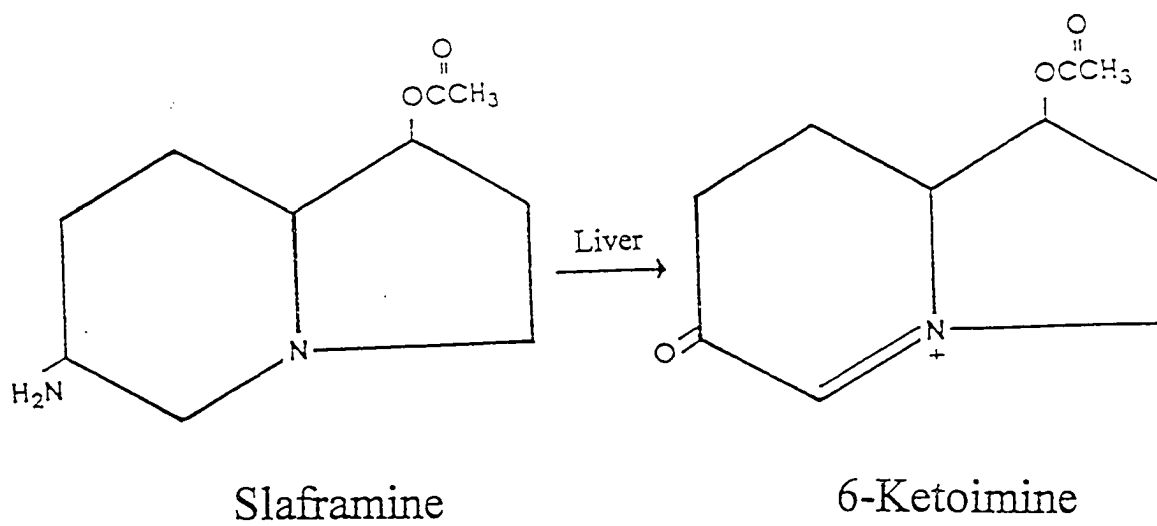
The active form of slaframine (see Figure 4), the 6-ketoimine compound, resembles acetylcholine: having two adjacent carbon atoms C4 and C8a separated by a quaternary nitrogen atom and an ethyl ester. The charged nitrogen part of the molecule is believed to be involved in binding the receptor, and the 6 keto group helps stabilize the nitrogen atom. The rest of the molecule must be involved in specificity, such as in the exocrine glands. This was suggested since in experiments with prepared active metabolite no binding to purified nicotinic-acetylcholine receptors occurred (34).

J. Similarities to Acetylcholine

This conformation provides a necessary requisite for binding to muscarinic acetylcholine receptors; resulting in parasympathomimetic action (10,34,35). Evidence that activated slaframine might have an action similar to acetylcholine is provided by the observation that atropine counteracts the effects of both the toxin and acetylcholine. Also, since both acetylcholine and phytostigmine cause salivation in mice, slaframine may have acetylcholine-like action (16).

To further investigate this relationship, guinea pig ileums were assayed to measure responses to slaframine relative to acetylcholine. A decrease in response to acetylcholine was observed after exposure of the pig ileum to activated slaframine. In addition, a rise in

Figure 4. The structure of slaframine and its metabolite 6-ketoimine.
Also, the structure of acetylcholine.



Acetylcholine

the resting level base line was seen; limiting use to five assays per pig ileum. When FMN-produced slaframine metabolite were used in the assay, a 100-fold increase in response was seen relative to assays using microsomal-produced slaframine metabolite. The activation of slaframine in the liver is aerobic whereas the photoreduction of FMN is anaerobic. Deamino-slaframine reduction of FMN is also anaerobic, but no such biological activity is seen. (36)

K. Active Metabolite

The active metabolite is very labile. Heating, raising the pH to 10, or lowering the pH to 2 results in a loss of activity. When using radioactive slaframine, no active metabolite was detected using thin layer chromatography; only slaframine and all starting materials were recovered. This suggested that the active species is transitory and readily converts back to slaframine.

The presence of the active metabolite could be shown by either microsomal or flavin activation. A rapid salivation response in rats was seen when an active slaframine metabolite produced by either FMN or flavinadeninedinucleotide (FAD) was injected. However, FMN was shown to be a more effective flavin than FAD (FMN was about 4 times faster) in producing slaframine's active metabolite. The active metabolite produced by FMN has been observed to have an apparent half-life of 8 minutes and decays at a first order rate. Similar results were obtained using microsomes. Since the half-life of the active species is 8 minutes, analysis must be performed within this time period.

The active metabolite has a somewhat unusual pharmacological action in that it produces a slow prolonged contraction in the ileum. This action lies between the action of acetylcholine and an anticholinesterase compound, such as eserine. Atropine, which reverses the effects of eserine, failed to do so when slaframine metabolite was present. Even with repeated washings of the ileum the contraction by the active metabolite of slaframine was not stopped. This result suggested a strong tight high-affinity binding at the muscarinic acetylcholine receptor. However, when atropine was given before slaframine metabolite no contraction occurred; reinforcing the similarity to cholinergic action. The binding of slaframine to these receptors must be greater than atropine, because high levels of atropine will not displace slaframine either in vitro or in vivo (10,34,36).

Slaframine is a potent cholinergic compound that stimulates the exocrine glands (11,34). Salivation is induced by two nervous systems 1) the cholinergic (parasympathomimetic) system; resulting in viscous secretion with the release of acetylcholine 2) the adrenergic (sympathetic) system; resulting in a watery type saliva and the release of adrenalin. Slaframine studies in cows resulted in a viscous saliva; suggesting that the toxin may be acting via the cholinergic system (3). Slaframine did not inhibit cholinesterase (11).

L. Review of Animal Studies

Over the years, many physiological effects caused by slaframine have been

reported and studied in various species. Early investigators, having witnessed field related effects of slaframine, began experimentation on a broad range of animals. A review of the animal responses to slaframine exposure follows: beginning with rodents and continuing with larger animals.

1. Guinea Pigs

Guinea pigs fed hay contaminated with slaframine began salivation within 48 hours. The severity of salivation peaks at 5 days, unless the animals refuse to eat. Depending upon the level of slaframine contamination in the hay, guinea pig responses ranged from severe weight loss and death to slight weight loss and slobbering.

Guinea pigs fed very toxic hays consumed more water and showed more enlargement of the lymph nodes than pigs fed hay that was only mildly toxic. Pigs fed highly toxic hay also showed general dehydration of tissues and abnormally small spleens and livers. Hemorrhage in the urinary tract and intestine were also seen in some cases. Histology showed an apparent dehydration and hyperactivity of the salivary glands (16). In general no response differences due to the sex of the pigs were noted (14).

Crump (37) described the salivation, lacrimation, urination, and defecation (SLUD) effect for parasympathomimetic responses consistent with the slaframine response. The saliva produced as a result of slaframine exposure was viscous and aerated. Lab animals exposed to slaframine became lethargic and have been known to develop tolerance levels. This may be related to enzyme induction: where metabolite conversion and degradation

occur at a more rapid rate (37).

Crump et al. (8,10) reported an LD₅₀ dose of 1 mg/kg in guinea pigs. This dose was given orally, SC, and IP. All pigs showed the clinical salivation response, but those given the IP dose died shortly after injection. Also a decrease in rectal temperature and respiration rate was observed in the guinea pigs (14).

Guinea pigs given atropine along with the toxin had no symptoms after five hours. Pigs given atropine two hours after the toxin had severe symptoms but no death. Atropine given four hours after the toxin had very little effect on relieving the symptoms of slaframine poisoning, but only one pig died. A guinea pig given a normally lethal dose of slaframine collapsed but returned to normal when given 8 mg atropine, SC. These results contradicted Aust's results, which showed that atropine could only block slaframine when administered before toxin (4).

When guinea pigs dosed with slaframine were given methantheline bromide (a muscarinic antagonist) the animals survived. When the drug was not given, the animals died. When hexamethonium (a nicotinic agonist) was given with the toxin, a 2.5 hour delay in slaframine symptoms was seen. Lab animals given antihistamines, especially phenothiazine derivatives like promethazine hydrochloride (an H₁-receptor antagonist), could survive otherwise lethal doses of slaframine (8,14).

No blood cholinesterase activity changes were observed in affected guinea pigs relative to control pigs (14). When pigs were given lethal doses of slaframine, their conjunctival, oral, and other tissues were extremely pale at post mortem. The vessels in

the thoracic and abdominal cavities were engorged with blood. The lungs were enlarged and the lung surfaces were pale and dry and contained small hemorrhagic areas. The lungs also contained a bloody foam exudate which was evident upon squeezing the tissue. Microscopically, the lungs showed large emphysemic areas and a disrupted alveolar structure. Small vessels in the lungs displayed a blood deficit while larger vessels were observed to be engorged. Abnormal levels of eosinophils were seen in the blood, lungs, and submucosa. The submucosal glands of the trachea were very active and contained many eosinophils. Pulmonary edema was seen in the lower sections of the lung. The liver was congested and, in many areas, displayed centrilobular necrosis. The kidneys were also congested (4,14). However, chronic exposures of slaframine (3 weeks) in guinea pigs showed no histological lesions (37).

2. Rats and Mice

Rats given 1 ml of crude filtrate by way of a stomach tube began salivating excessively after 90 minutes (14). Unlike guinea pigs, rats did not show clinical signs of lacrimation (37).

Maseehudin and Nichols (38) conducted a slaframine study utilizing 217 rats and 122 mice; preparing slaframine according to the method of Crump et al. Their doses were specified as a minimum salivary dose (MSD) of mat (*Rhizoctonia leguminicola*) extract. The MSD was determined separately for each batch of mat material, because the unpurified slaframine existed at differing levels within each mat.

Rats given an MSD orally showed no change in RBC, WBC, or hemoglobin, but neutrophils were increased three fold. Lymphocytes were depressed and clotting and prothrombin times were decreased. Urea, uric acid, alkaline phosphatase, and serum GOT levels in the blood increased. Calcium, phosphate, cholesterol, bilirubin, total protein, albumin, and LDH levels in the blood did not change. An oral MSD of slaframine also resulted in a decrease of oxygen consumption in the brain and kidney and an increase salivary and pancreatic activity, but no change in the liver. Gastric acidity and bile flow were also increased. Abortion, uterine hemorrhage, and death were observed in 19 day-old pregnant rats at a generally sublethal MSD given orally (4,38).

As a result when 2 MSD was administered orally, heart rate, rectal temperature, and respiration rate were unchanged in rats. Pentobarbital-induced sleep times were increased only when twice the MSD was given (38).

Other investigations at varying dosages showed an increase in the contractions of both the uterus in mice and the colon in rats, and milk let-down in mice was stimulated two-fold (38).

Aust (39) found the salivary glands and the pancreas in rats to be most susceptible to slaframine given at a dose of 0.6 mg/kg. The prolonged activity in these glands could have been due either to activation by the liver or to the possibility that the toxin has a high affinity for the receptors located there. This may indicate the uniqueness of the receptors of these glands. The pancreas weight of the rats dropped to 50% that of controls. The amount of extractable protein from the pancreas decreased rapidly but

returned to normal levels five hours after the injection of the dose. At this time the pancreas weight also returned to near normal. The chymotrypsin levels of the pancreas decreased to insignificant levels. A similar decrease was seen for all digestive enzymes analyzed. This would suggest that slaframine causes the gland to secrete all the enzymes stored and then to begin synthesis of new enzymes.

3. Cats

In another study by Aust (3,5,10,12,33), an anesthetized cat was given an IV dose of 0.3 mg/kg slaframine. The submaxillary duct of the salivary gland was cannulated to measure salivary flow. Salivation was observed to begin within 30-40 minutes after the administration of the toxin, and continued for a six hour period. Maximum salivation occurred after a one-hour time period. Cats were unable to respond to a second dose of slaframine (1).

All the exocrine glands, including the pancreas, were stimulated, and excessive lacrimation and diarrhea also occurred (1,33). But Aust (12,40) observed no effect on heart rate, blood pressure, ganglionic transmission, or respiration. These observations contradicted the work of Crump et al. (14), Edwards and Crump (12), Maseehudin and Nichols (38), and Sapio and Smalley (12); who all observed a decreased heart rate and respiration in studies involving the effects of slaframine on animals.

4. Chickens

Crump et al. (14) gave chickens 5 ml *Rhizoctonia leguminicola* culture filtrate. Salivation began after one hour, and the chickens appeared somnoient and defecated frequently, but recovered after several hours.

Johnson et al. (8) reported an LD50 for broiler chicks at 81.6 mg/kg. This high LD50 for chicks relative to other species suggested a difference in susceptibility among species. This study also showed intoxicated broiler chicks to have increased nonesterified fatty acids and variable insulin levels.

Froetschel et al. (10) reported an LD50 of 11 mg/kg for 120-day-old broiler chicks. Perhaps age or type of chicks influence these differences in LD50 levels. Froetschel et al. (41) also reported that broiler chicks given slaframine at a dose of 1 mg/kg showed a 21.4% increase in plasma glucose after one hour. Growth hormone levels were also shown to increase 449% and 948% at 8 and 12 hours, respectively. Perhaps hormonal secretions of this type are affected because the endocrine glands are innervated by the parasympathetic neurons.

In another study, Froetschel et al. (42) looked at slaframine's effects on digestive enzymes in 240-day-old broiler chicks. Separate groups of birds were fed 0, 8.9, 17.9 µg slaframine/kg body weight for 21 days. Body weight, feed intake, and excreta output were not affected by slaframine. However, the liver weight decreased, and pancreas weight increased as a result of slaframine intoxication. Lipase activity decreased in chicks given the 17.9 µg dose. No difference was seen for other digestive enzymes, but an

increase in protein was seen for all birds given slaframine relative to the control population.

Hagler (8) found slaframine did not effect plasma cholinesterase levels in chicks. This supported the theory that slaframine action is at the muscarinic receptor.

5. Swine

Crump et al. (14) also studied the effects of slaframine on swine. One pig was given 200 ml *Rhizoctonia leguminicola* mat extract and another pig was given 200 ml of a crude filtrate (blended mat material) by stomach tube. The pig given the extract had excessive salivation, coughed, vomited, defecated, and urinated. Respiratory difficulties caused the pig to collapse. The skin was cyanotic. When this pig was given 8 mg atropine SC it began breathing normally and fully recovered within hours. The pig given the filtrate had less severe symptoms and recovered without treatment.

Swine intoxicated with slaframine in addition to a drop in respiration also showed a drop in heart rate (12). When slaframine was administered to fasted gilts no effect on circulating insulin was seen but there was an increase in nonesterified fatty acids relative to pigs in the control group (8).

6. Horses

While no controlled experiments on the effects of slaframine in horses are reported, field effects have been seen in cases where horses have consumed feed or hay containing

Rhizoctonia leguminicola. The clinical symptoms observed included excessive salivation, anorexia, lacrimation, diarrhea, polyuria, bloating and stiffness. In one case, abortion was associated with the slaframine intoxication (4,6,43).

7. Ruminants

Ruminants are the largest group of animals affected by slaframine toxicity. This is due to their extensive field exposure to contaminated feedstuffs, which are the source of the problem. Because of this, a large number of experiments have been done on the effects of slaframine intoxication in ruminants.

When a goat, a ewe, and a calf were given 0.1-0.2 mg/kg IP dose of slaframine, all showed an increase in pancreatic flow after a delay period (the delay was, presumably, caused by the necessary activation of slaframine) (3,8,12,33,35,39). In the goat and ewe the increased pancreatic flow lasted for several hours. The increase lasted ten hours in the calf (12,33). The pancreatic flow volume in the goat increased two-fold. The protein content of the pancreatic fluid increased three-fold initially but then rapidly returned to normal (11).

In calves the pancreatic duct is independent of the bile duct so pancreatic fluid can be collected. The protein content, pH, and viscosity of the pancreatic fluid were not changed significantly by slaframine intoxication (8,39). However, the mucoprotein content (based on the N-acetylneuraminic acid (NANA) content) decreased approximately 50% from normal. There was no change in the bile flow rate.

An increase in enzymatic activity (protein content) along with an increase in pancreas weight was also observed (39). In the goats an increase in the specific activity of trypsin suggested an increase in the production of digestive enzymes (10). In the calf and the goat new protein synthesis was shown by the incorporation of ^{14}C leucine into the protein fraction (10,11,39). It is not known if slaframine similarly effects the secretions of the intestine (8).

Crump et al. (4,14) gave steers an oral dose of a crude filtrate (blended mat material) of *Rhizoctonia leguminicola*. Salivation occurred within approximately 30 minutes. Lacrimation occurred from time to time, and the salivation intensified at 24 hours. At this time the animals ceased to eat, urinated frequently and developed watery diarrhea. The symptoms began to diminish at 48 hours, and the animals recovered by 96 hours. In dairy cattle a decrease in milk production and body weight is also associated with slaframine toxicity. Steers given an injected slaframine dose of 18-24 $\mu\text{g/kg}$ body weight showed increased levels of growth hormone, glucagon, and inulin in their blood (8).

Crump et al. (14) also gave a ewe some of the crude filtrate (blended mat material) by stomach tube. The ewe began salivating within one hour and developed open mouth breathing, becoming cyanotic. The ewe was observed to be near death, was given 8 mg atropine, and recovered. No morphological tissue changes have been described in either sheep or cattle (4).

The most recent work in the literature on slaframine intoxication has concentrated

on the potential benefits of the buffering effect that results from excessive salivation in ruminants. Froetschel et al. (10,44) gave rumen cannulated wethers 0, 25, 50, or 100 gm/day of red clover feed pellets containing 7 µg/g slaframine. An increase in salivary flow ranging from 5-446% was observed. A 15-37% increase in the acetate/propionate ratio and a 4-18% increase in rumen dilution rate were observed. When purified slaframine was given SC to steers the salivary flow increased 316%. These findings suggest slaframine may be useful in treating digestive disorders such as feeding high grain restrictive roughage diets.

Since it was established that slaframine increases salivary flow in ruminants, composition of saliva has been of concern. Using steers given an intermuscular (IM) dose at 0, 6, 12, and 24 µg slaframine/kg body weight, Froetschel et al. (45) found that salivary flow increased up to 68.6%. Salivary sodium content increased and potassium content decreased. Phosphorus concentration was unchanged, so the output of phosphorus increased as salivation increased. The protein content increased at the 12 µg/kg dose but decreased at the higher dose of 24 µg/kg. There was a decrease in the NANA mucoprotein constituent, probably due to dilution. There was no change in pH, osmolality, or buffer capacity with slaframine intoxication. Slaframine could be influencing the ruminal buffering capacity and fluid turnover by increasing in salivary flow.

Froetschel et al. (46) also looked at the effect of slaframine on rumen motility. In both the wethers and the steers dosed with slaframine the ruminal pressures were

decreased. Both the primary and secondary contraction frequencies decreased. Inhibition of ruminal motility has also been reported for acetylcholine and other muscarinic agonists. Froetschel suggests that the release of acetylcholine may release catecholamines, causing less motility by an endocrine mechanism since adrenalectomized animals show no decrease in motility when given acetylcholine. Slaframine has been shown to increase growth hormone and glucagon levels when motility is inhibited. Gastrointestinal (GI) peptides and hormones have also been shown to inhibit ruminal motility. Perhaps the ability of slaframine to cause salivation and decrease motility indicates that different types of receptors are involved. Possibly slaframine affects both kinds of receptors (those in the GI tract and the exocrine glands), because dose level responses for each type of receptor have a different threshold at each site.

Froetschel et al. (47) continued their research by using slaframine to manipulate the aspects of digestion such as flow, motility, and fermentation, in sheep and cattle. The ruminal dilution rate of the wethers and steers was unaffected at a 24 µg/kg dose; however, the volume and outflow both increased. These increases are probably due to the increase in salivation, even with the decrease in ruminal contractions. The pH of the rumen increased in those animals given slaframine and the volatile fatty acids decreased. Thus, ruminal buffering may be a result of slaframine. Along these lines, Jacques et al. (48) found that ruminal flow increased in sheep but not in steers. However, similar to Froetschel et al. (10,44), there were increases in ruminal outflow and dilution rate. An increase in feed and forage intake alters the ruminal fluid balance. With the use of

slaframine one can alter saliva levels but cannot effect feed or water intake.

In further experiments, Froetschel et al. (49) found that saliva increased 30-40% and that the ruminal and abdominal outflow increased in the slaframine treated animals. In previous experiments it was observed that slaframine did not affect the fermentation end products of buffering capacity. However, this could be due to the frequent feeding (which is known to increase pH, the acetate/propionate ratio, and ammonia in the rumen, and also to influence the end products) done in this experiment. There was an increase in post ruminal digestion in the slaframine treated animals. Starch digestion increased with the level of slaframine. The microbial protein outflow was higher in the slaframine animals; indicating microbial efficiency. A positive correlation between salivation and bacterial protein synthesis resulted in animals given slaframine, and a shift in digestion sites from rumen to the lower digestive tract occurred.

Kelly et al. (40) looked at slaframine as an influence on the shifting of ruminal digestion in cows by looking at the role of the reticulo-omasal orifice. The reticulo-omasal orifice plays a role in the regulation of digestion from the rumen to the lower digestion tract. Slaframine increased the opening of the orifice 30-33% over controls and reticular contractions decreased. This agrees with the previous data on cholinergics; which show an increase in the rate of fluid and the passage of particulates in the rumen. Slaframine prolonged the orifice opening 38% and 25%, respectively, for 20 and 40 µg/kg body weight doses. The increase of the opening and the decrease in rumen motility may be due to the presence of different receptor subtypes, or, possibly, slaframine could be

acting indirectly by releasing neuropeptides.

Slaframine could be of assistance to animals on high-grain low-roughage diets by providing a more efficient mixing of saliva and rumen contents for bacterial protein digestion. Perhaps slaframine could assist animals on high roughage diets by increasing reticulo-omasal orifice opening time to move large mass material through the rumen; decreasing transit time and increasing intake.

M. This Contribution

This research was undertaken to provide a preliminary pharmacokinetic study of slaframine in lactating goats. The primary goal of this work was to determine whether slaframine can be transferred into milk, and, as a result move into this food source to nursing goats and humans. The results of this work show that slaframine is deposited into the milk in measurable levels. The study also investigated the rate of elimination through plasma and milk. Estimates of the half-life for the elimination of slaframine from lactating goats were also determined.

The study required the development of a more sensitive methodology for the extraction and detection of slaframine in saliva, plasma, and milk. A very sensitive method of detecting slaframine was developed using fluorescamine, a fluorescent derivative for amines, and HPLC analysis of this derivative.

The remainder of this dissertation outlines the work done and provides a discussion of the conclusions reached. Section two. Materials and Methods, outlines the procedures

and methodology used in this work. The new methods developed in the course of this work are included in this section. Section three, Results and Discussion, provide an analysis of the data gathered and a discussion of the implications thereof. Section four, Summary, reviews the work as a whole.

In the following discussions, all references to "water" refer to water prepared through a Millipore® cartridge system. All solvents used were either HPLC-grade or ACS reagent-grade. A listing of all supplies used and their sources is included as Appendix C. A list of abbreviations used in the text is included as Appendix D.

II. MATERIALS AND METHODS

A. Growth of the Organism

Rhizoctonia leguminicola 26280 was obtained from the American Type Culture Collection (ATCC). Approximately 5 ml of sterile water was added to the freeze dried culture and mixed. The culture suspension was aseptically pipetted onto Saboraud-dextrose (SAB-DEX) slants. The culture slants were incubated for two weeks and then the organism was transferred aseptically with a hooked culture wire onto new slants to maintain the culture. The lids of all slants were loose to allow air exchange for culture growth. After two weeks, slant media and growth were aseptically transferred to a 500-ml sterile glass blender containing 75 ml of sterile water and blended for one minute. One ml of this culture suspension was aseptically transferred to each of 60 SAB-DEX petri plates. The plates were allowed to grow for 8 days under Sylvania cool white fluorescent tubes. Mycelial mats were collected from the petri plates and adherent agar was scraped from the mats.

B. Extraction and Purification of Slaframine

The mats were extracted in a Soxhlet apparatus for approximately 15 hours using 800 ml of 95% ethanol. The ethanol was allowed to cool to room temperature. The ethanol was then divided into two fractions of 400 ml and each placed in 1000-ml beakers. Three hundred ml of water was added to each ethanol fraction and shaken. The

pH of each fraction was adjusted to 2.0 with 2N HCL. Each fraction was then extracted four times with 100 ml of methylene chloride each time in a 1-liter separatory funnel. The resulting methylene chloride from the total of eight extractions was discarded.

Then 200-ml saturated sodium chloride solution was added to the remaining aqueous portion of each of the two separatory funnels and mixed. Each fraction was placed in a 1000-ml beaker. The pH of both fractions was adjusted to 10.0 with 10% sodium carbonate. These fractions were again extracted four times in 1-liter separatory funnels with 100 ml of methylene chloride for each extraction. All methylene chloride (800 ml) was saved and placed into a 2-liter round-bottom flask. The methylene chloride was roto-evaporated at 58 °C to near dryness. The residue from the flask was redissolved in 10 ml of HPLC-grade chloroform and transferred into a graduated tube.

To further purify the extract, 1 ml from the 10 ml of chloroform was streaked onto a 10x20 cm reverse-phase TLC plate about 2 cm in from each side edge of the plate. A 0.5- μ g slaframine standard was spotted about 1 cm in from each side edge of the same plate. The reverse-phase TLC plate was developed in ethanol/water/glacial acetic acid (65/35/1, v/v/v) to the top of the plate. Using a glass cutter, the TLC plate side ends were cut approximately 2.5 cm in from both sides of the plate. The cut parts of the TLC plate were placed in a TLC tank containing iodine fumes for about 10-15 minutes or until standards of slaframine and bands from extract are visible. The cut parts were then placed next to the TLC plate and the area corresponding to the slaframine standard band on the unexposed portion of the plate was marked using a pencil. The TLC coating in the

marked band was scraped from the exposed plate. This coating was placed in a 250-ml Erlenmeyer flask. This procedure was repeated for all 10 ml of chloroform extract.

One hundred ml of HPLC-grade methanol was added to the flask containing the TLC coating. The contents were mixed and the flask was placed at -10°C for 15 hours. The methanol mixture was then removed from the flask and placed in a 200-ml round-bottom flask and the methanol was roto-evaporated at 58 °C to dryness. The resulting purified film was redissolved from the round bottom flask in 10 ml of HPLC-grade chloroform and transferred to a graduated tube.

Using a 10-microliter syringe, one microliter was removed from the 10 ml of chloroform purified extract and placed in each of three 2-dram vials. The fluorescamine derivatives (see section C) for each vial were prepared and analyzed to determine the concentration by the HPLC procedure (section D) against a known derivatized slaframine standard. The average of three determinations of the unknown chloroform extract was calculated.

C. Preparation of the Fluorescamine Derivative for HPLC Analysis

In all instances where fluorescamine derivatives were used, 100 microliters of a 0.025M sodium borate + 0.1M HCl pH 8.5 buffer was added to a dried residue of standard, sample, or spiked sample and mixed with a vortex mixer. Then 100 microliters of fluorescamine solution at a concentration of 6 mg/25 ml in reagent-grade acetone was added and vortex mixed. This mixture was concentrated to dryness by nitrogen effusion

with low heat. The resulting fluorescamine derivative was used for HPLC analysis.

D. HPLC Specifications for Fluorescamine Derivative Analysis

A Hamilton PRP-1 reverse phase 10 μ m 250 x 4.1 mm HPLC column with a mobile phase of acetonitrile/20mM sodium borate 10mM triethylamine pH 12.0 (35/65, v/v) and a flow rate of 1 ml/min was used for all HPLC analyses. A McPherson SF-749 fluorescence detector with an HSA assembly was used with the excitation level was at the 365 nm mercury line and the emission used a CF 400 filter. The sensitivity range was set at 0.3 with a time constant of 5.0, and the suppression background set to low. The gain (pmt/Hv) was set at 490. The mobile phase was pumped by a Waters 6000A HPLC pump and a Shimadzu Sil 9A autosampler was used for injection of samples. Samples were injected at 10 microliters and spiked samples and standards were injected at 5 microliters. The readout device used was a Shimadzu CR501 Chromatopac® integrator set with an attenuation of 5.0 and a speed of 2 mm/min.

E. UV Spectral Analysis

A 20-microliter volume of the purified slaframine in chloroform (created in section B) was placed in a 2-dram vial. This was concentrated to dryness by nitrogen effusion and a 200 μ l of HPLC-grade methanol was added. A 20- μ l volume of the methanol fraction was injected into a 3x3 pecosphere C-18 HPLC column. The mobile phase was methanol/water (50/50, v/v), at a flow rate of 1 ml/min. and was monitored with a Waters

551 diode array detector at 260 nm. The absorbance of the slaframine peak was measured and the concentration was calculated using Beer's Law.

F. Preparation of the Dose

The dosage used for the goats in this experiment was 0.05 mg/kg. The amount of toxin needed for the dose was measured from the chloroform concentrate which had been checked by using the UV spectral analysis procedure (section E) a day prior to dosing. Approximately 30 minutes prior to dosing, the measured chloroform toxin was placed in a 50-ml beaker and concentrated to dryness by nitrogen effusion under low heat. A 0.45- μ m Costar® sterile syringe filter was prewashed with 2 ml of sterile saline. Then 2 ml of sterile saline was added to the 50-ml beaker containing the dried slaframine, and it was dissolved by stirring. The prewashed filter was attached to a 5-ml syringe with needle and the toxin solution was poured from the beaker into the syringe barrel. Using the syringe plunger the toxin solution was pushed through the filter and into a sterilized septum vial. This procedure was repeated again with another 2 ml of sterile saline solution to rinse the beaker and placed in the same septum vial. The vial was stored on ice until the dose was given.

G. Insertion of Catheter

The left side of the neck of the goat was first shaved to provide an unobstructed area for catheter insertion. The shaved area was disinfected with Nolvasan® surgical

scrub and then with alcohol; the excess was removed with sterile gauze. A 2-cc injection of Procaine was then given SC to anesthetize the area. A 16-gauge needle was inserted through the skin to provide a hole for the catheter. A 14-gauge, 5-inch angiocatheter was inserted through this hole and into the left jugular vein. To secure the catheter, 4 inches of white adhesive tape was wrapped approximately 2 cm from the top of the catheter with the catheter in the middle of the tape. To secure the catheter in place the tape ends were stitched to the goats skin. The catheter was flushed with 8 ml of sterile heparinized solution.

H. Dosing the Animals

The dose was removed from the sterile septum vial into a sterile syringe. The dose was then given into the right jugular using an 18-gauge needle attached to a 12-ml syringe containing the toxin dose in sterile saline. The dose was given in this manner so as not to interfere in the collection of blood samples. The full dose was slowly injected into the goat (IV bolus). Upon completion this was called zero time.

I. Collection of Blood Samples

Blood samples were taken from the first goat at zero time; zero plus 2.5, 5, 10, 15, 30, and 45 minutes and 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 24, and 48 hours. The remaining four goats were sampled on the same schedule, but only up to 5 hours. At each sampling time, approximately 3 ml of blood was removed from the catheter and discarded to eliminate

dilution or traces of the heparin prior to blood sample collection. Two 10-ml blood samples were then removed from the catheter using a 18-gauge needle attached to a 12-ml syringe. The catheter was flushed with 8 ml of a heparin solution following blood collection to keep the catheter open.

Each 10-ml blood sample was then placed in 10-ml EDTA vacutainer tubes. The EDTA tubes containing the blood samples were immediately inverted several times to mix the blood with the EDTA to avoid clotting. The samples were then refrigerated.

When the sampling period was completed for each goat, all blood samples for that goat were centrifuged in a clinical centrifuge for 15-30 minutes, and the plasma was removed. Two-ml aliquots of plasma were placed in 10-ml test tubes (2057 Falcon), labeled with the goat number and the sample time, and then frozen at -10 °C.

J. Collection of Milk Samples

Each goat was stripped of all milk immediately prior to dosing. This stripping of milk was identified as the zero-time sample. Milk samples were collected from the first goat at zero plus 30 minutes 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 24, 31.5, and 48 hours. The remaining goats were sampled at zero, 15, 30, and 45 minutes and 1, 1.5, 2, 3, 4, 5, and 24 hours. At each collection time the goat was totally stripped of milk to avoid the possible dilution of toxin transferred to the milk during the following interval. (50,51) The entire sample from each collection was immediately refrigerated. Upon completion of the collection period all milk sample volumes were measured and recorded and aliquots

containing 3-5 ml (wherever possible) were placed in 10-ml test tubes (2057 Falcon). The test tubes were labeled with the goat number and time of collection and frozen at -10 °C.

K. Collection of Saliva Samples

Saliva samples were not taken at regular intervals because of time constraints resulting from other, more primary, collection activities and because of differences in salivation levels among the goats. Samples were collected, when possible, on 30 minute to 1 hour intervals. Saliva was collected manually by holding a cup under the goat's mouth and catching the saliva. Upon completion of the collection period, each sample's volume was measured and recorded along with the time of collection. Saliva was then measured into aliquots of 3-5 ml, transferred into 10-ml test tubes (2057 Falcon) (with the reserve, if any, placed in a whirl pack), and were frozen at -10 °C.

L. Method for the Extraction of Plasma

A 1-ml volume of zero-time plasma was added to each of four separate 15-ml screw-cap glass test tubes. Each of these four fractions had one of the following amounts of slaframine added: 0, 0.05, 0.1, and 0.2 µg. A 1-ml volume of each timed plasma sample was added to a separate 15-ml screw-cap glass test tube. All tubes were extracted using the following procedure:

Two ml of 2N HCl and 2 ml of methylene chloride were added to each test tube. The tubes were then vortex mixed and centrifuged at 2200 rpm for 15 minutes. The top

aqueous layer from each tube was then removed and placed in a 10-ml beaker. A 3-ml volume of 1N NaOH solution was added and the pH was adjusted to 10 with 10% sodium carbonate. The contents of each 10-ml beaker was poured into a clean 15-ml screw-cap glass test tube. The contents of each test tube was extracted with 2 ml of methylene chloride and centrifuged at 2200 rpm for 15 minutes. Then this extraction was repeated.

The bottom methylene chloride layer from both extractions was removed and passed through a pasteur pipette sodium sulfate column. The methylene chloride extracted from each test tube was collected into a single 2-dram glass vial. The methylene chloride in each 2-dram vial was concentrated to dryness by nitrogen effusion with low heat.

The fluorecamine derivative (see section C, above) of the slaframine for each extracted blood sample was prepared. A 0.1- μ g slaframine standard and a fluorecamine blank were also prepared. Each sample was resolvated in 0.2 ml of acetonitrile/water (35/65, v/v). The standards and spiked samples were resolvated in 0.5 ml of acetonitrile/water (35/65, v/v). Samples, standards, and spiked samples were analyzed by HPLC (see section D).

M. Method for the Extraction of Milk

For each timed sample, a C-18 Sep-Pak® cartridge was prepared by forcing 4 ml of methanol then 4 ml of water through the cartridge with a syringe. An aliquot containing 2 ml of milk and 2 ml of water was forced through the prepared C-18 Sep-Pak® cartridge and the effluent was discarded. A 5-ml aliquot of water was then forced

through the C-18 Sep-Pak® and the effluent was discarded. Finally, 30 ml of methanol/water (75/25, v/v) was forced through the C-18 Sep-Pak® and the effluent was collected in a 100-ml beaker.

A 25-ml aliquot of water and a 3-ml aliquot of saturated sodium chloride solution were added to the beaker. The pH of this solution was adjusted to 10.0 with 10% sodium carbonate. The solution in the beaker was placed into a 125-ml separatory funnel and extracted two times each with 10 ml of methylene chloride.

The methylene chloride fractions were combined into a 50-ml beaker and concentrated to approximately 4 ml by nitrogen effusion with low heat. The concentrated methylene chloride was passed through a pasteur pipette sodium sulfate column and collected into a 2-dram vial. The beaker was rinsed with 2 ml of methylene chloride two times. The methylene chloride was passed again through a pasteur pipette sodium sulfate column and collected into a 2-dram vial. The methylene chloride in the 2-dram vial was evaporated to dryness under nitrogen effusion with low heat.

The fluorecamine derivatives (see section C. above) of the extracted slaframine in the 2-dram vials were prepared. The samples were resolvated in 0.2 ml of acetonitrile/water (35/65, v/v) and the standards and spiked samples were resolvated in 0.5 ml of acetonitrile/water (35/65, v/v). A 0.1- μ g slaframine standard and fluorecamine blank were prepared with the samples.

A standard curve was prepared using the same C-18 Sep-Pak® procedure above with four 2-ml zero-time stripped milk samples containing 0, 0.05, 0.1, and 0.2 μ g of

slaframine. The prepared standard curve and all milk samples were analyzed by HPLC (see section D).

N. Method for the Extraction of Saliva

Two duplicate 1-ml aliquots of saliva for each collection interval were added to a 15-ml screw-cap glass test tubes. A 0.1- μ g slaframine standard was added to one of each set of duplicate tubes as a spiked sample.

To each tube, 2 ml of 2N HCl and 2 ml of methylene chloride were added, vortex mixed, and centrifuged at 2200 rpm for 15 minutes. The top aqueous layer was removed and placed into a 10-ml beaker. A 3-ml aliquot of 1N NaOH and a 1-ml aliquot of saturated sodium chloride solution were added to the beaker. The pH was adjusted to 10.0 with 10% sodium carbonate.

The contents of each beaker was poured into a 15-ml screw-cap glass test tube. The contents of each test tube was extracted with 2 ml of methylene chloride. The methylene chloride was added, shaken by hand for 1 minute, and then centrifuged at 2200 rpm for 15 minutes. The bottom methylene chloride layer was removed. This extraction was performed twice for each beaker. The combined methylene chloride from both extractions was passed through a pasteur pipette sodium sulfate column and collected into a 2-dram vial. The methylene chloride fractions were concentrated to dryness by nitrogen effusion and low heat.

The fluorescamine derivatives (see section C, above) were prepared for all samples

and spiked samples. A 0.1- μ g slaframine standard and a fluorescamine blank were also prepared. The samples were resolvated with 0.2 ml of acetonitrile/water (35/65, v/v) and the standards and spiked samples were resolvated with 0.5 ml of acetonitrile/water (35/65, v/v). All saliva samples were analyzed by HPLC (see section D).

G. Statistics and Computer Analysis

To extract the pharmacokinetic data, the PC-based program PCNONLIN 4.2 was used. The following programs were used to analyze the plasma data for all five goats individually and for the mean of all five goats. All the models used were for IV bolus input.

Model 1 is for a one compartment model. Models 7 and 8 are for two compartment models. Model 18 is for a three compartment model. The difference between models 7 and 8 is that model 7 uses microconstants as the primary parameters while model 8 uses macroconstants as the primary parameters.

Significance tests of the estimated parameters and standard errors obtained using PCNONLIN 4.2 were done using Student's t distribution at probability of less than 0.05 (52).

Sigma Plot 5.0 was used for linear regression. This program uses a rational polynomial to compute the t values for confidence limits. Standard errors of the mean values were also computed using this program. The standard deviation was calculated for the data from the method evaluation by solving for sigma for a sample population (52).

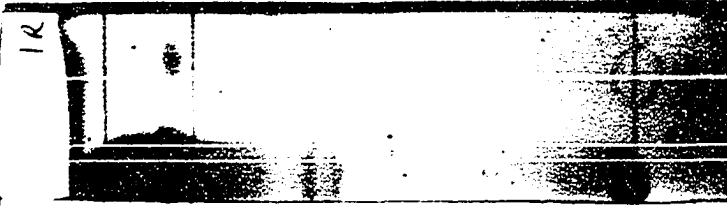
III. RESULTS AND DISCUSSION

A. Purification of Slaframine

Rhizoctonia leguminicola was grown as described in methods section (C), and slaframine was purified from the culture mat material as described in methods section (D). The purification procedure for slaframine was modified from the Aust (5,14) and Crump (11,12) procedures. The culture mat material was extracted as in the Aust procedure, but extraction time was decreased to 15 hours. Water was added to the ethanol extract, and the extract was made acidic (similar to the Crump procedure), but methylene chloride was used to remove the fat soluble and acidic soluble impurities instead of petroleum ether, (which is not as effective at removing the acidic soluble impurities). As in both procedures, the pH was raised to 10 for the extraction of the alkaloid, slaframine, into methylene chloride. In the procedure developed here, however, a saturated sodium chloride solution was added to enhance the recovery of the analyte.

Further purification of slaframine was done using reverse phase thin layer chromatography and visualization with iodine fumes. This was based on previous work by this investigator (53). An example of a typical TLC plate utilized in this process is shown in Figure 5. The TLC coating containing the slaframine was removed after having been identified using the iodine visualized slaframine standards. The pure slaframine was eluted from the reverse phase coating by soaking it in HPLC methanol as stated in methods section (B). The methanol was removed by roto-evaporation and the slaframine

Figure 5. TLC purification of slaframine
s = 0.5 μ g slaframine standard
e = part of streaked slaframine extract
A = developed in iodine fumes
B = undeveloped

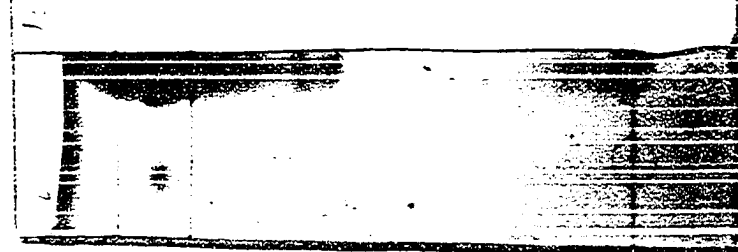


1R

1R

S
S

A



S
S

A

B

was then resuspended in HPLC chloroform. Table 1 illustrates the yields of the various steps in this purification scheme. Only a 17% loss was seen between the first two steps. Overall, 40% of the pure slaframine was recovered by the TLC procedure. The 60% loss was probably due to poor recovery of the slaframine bound to the C-18 phase of the TLC coating. This procedure resulted in a known consistency and purity of the isolated slaframine, providing the ability to create a reproducible product.

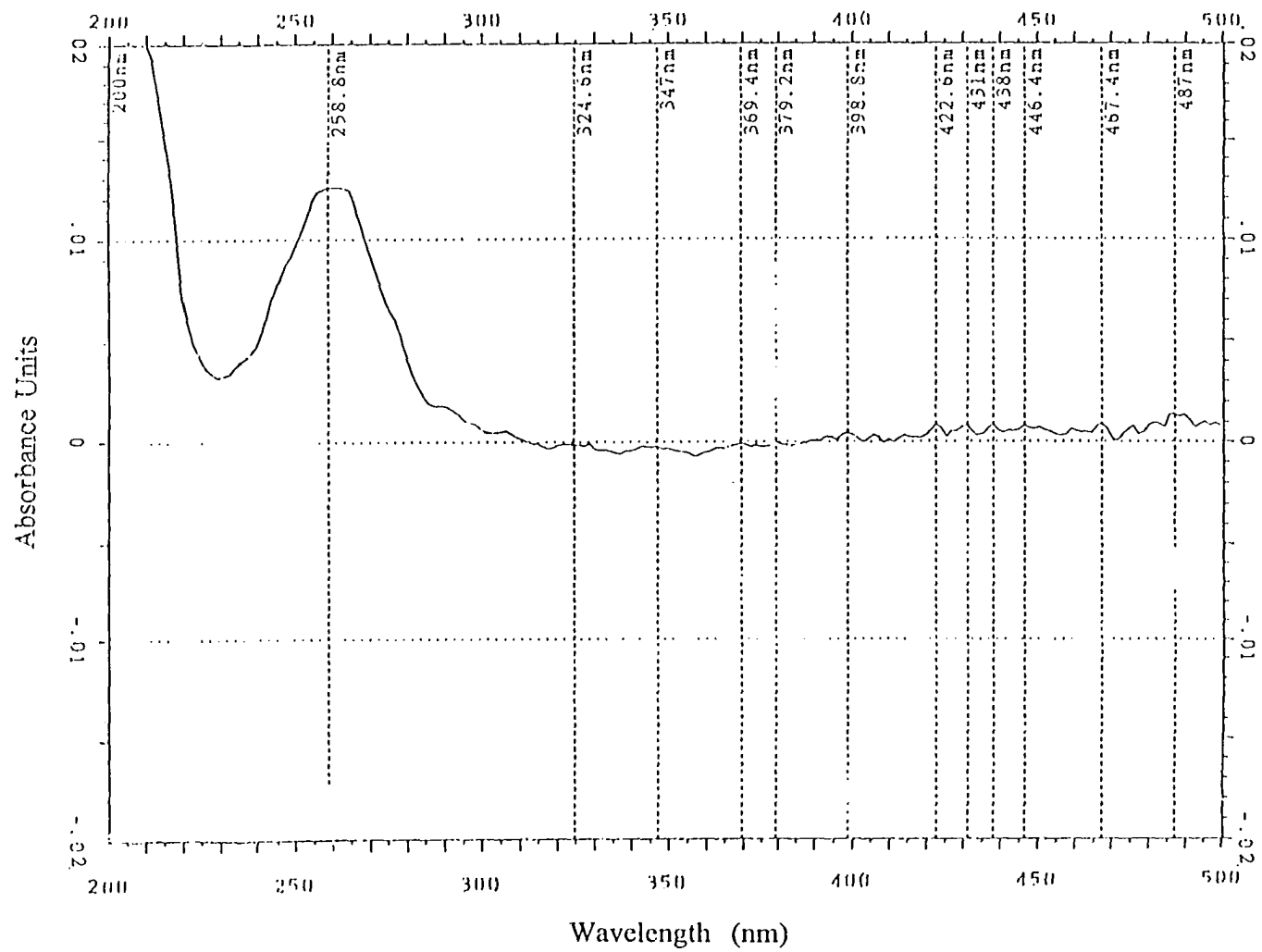
Table 1. Purification of slaframine from *Rhizoctonia leguminicola*

Purification steps	mg slaframine/60 mats	% yield
Soxhlet extraction	18.0	100
Liquid/liquid extraction	15.0	83
TLC purified	7.2	40

The slaframine was stored at -10 °C in HPLC chloroform for further use. This was done because producing the picrate for such small amounts of toxin resulted in unacceptable losses in the recovery of slaframine.

The purity of the slaframine was checked by UV-diode array detection. Figure 6 shows an example of a characteristic spectrum of the pure slaframine toxin. A known volume of the purified slaframine was chromatographed by HPLC (see methods section E). The slaframine eluted within 0.5 minutes and the spectrum shows one peak (at approximately 259 nm) denoting the purity. Using Beer's Law, the concentration of the

Figure 6. The UV-diode array spectra of HPLC peak of slaframine fraction.



slaframine was determined for the purified fractions. Aust (13) reported that slaframine absorbed at 260 nm with a molecular absorptivity of 48. The concentration was then calculated as follows:

$$\begin{aligned}
 &= 0.0125 \text{ (absorbance units)} \div 48 \text{ (molecular absorptivity)} \times 1 = \text{mole} / \text{l} \\
 &= 0.0002604 \text{ mole} / \text{l} \times 198 \text{ (molecular weight of slaframine)} \text{ g} / \text{mole} \\
 &= 0.052 \text{ g} / \text{l} \text{ or } \mu\text{g} / \mu\text{l} \times 10 \text{ (dilution factor)} \\
 &= 0.52 \text{ } \mu\text{g} / \mu\text{l} \text{ in the slaframine fraction}
 \end{aligned}$$

This method of determining slaframine concentration provided good results when compared to a purified slaframine sample obtained as a gift from H. Broquist (Vanderbilt University, Nashville, TN). The concentration of the newly purified slaframine determined by UV-diode array methods compared favorably to the H. Broquist slaframine concentration when compared by gas chromatography methods, where both methods used acetate derivatives of the slaframine (19). The concentration from the UV-diode array method was also compared using the method of Stahr (54) which used a weighed quantity of purified swainsonine and quantitated slaframine by peak height comparisons of the acetate derivatives of swainsonine and slaframine using gas chromatography.

B. Slaframine HPLC Analysis

In this study the parent toxin, slaframine, was analyzed; not the active metabolite

of slaframine (6-ketoimine). The active metabolite has a half-life of 8 minutes. Current technology cannot analyze for the metabolite within the short time frame that this half-life provides. This was not a problem for this research, because the slaframine metabolite is transitory in nature and converts back to slaframine (10,51,55).

Because relatively low levels of slaframine need to be identified in a pharmacokinetic study, a new and more sensitive methodology for slaframine analysis was developed for this research. Earlier work by this investigator using reverse-phase TLC showed that a fluorescamine derivative could be made by spraying the TLC plate with base and then with fluorescamine (56). This knowledge led to the development of a more sensitive HPLC method for slaframine detection.

This method displayed several advantages. By using a fluorescent derivatizing agent, fluorescamine, a 10-fold increase in sensitivity can be achieved over previous reported methods. Since slaframine contains a primary amine function a fluorescamine derivative can easily be made. The fluorescamine derivative is a stable derivative.

The derivative is formed in a basic environment between pH 8 and pH 9. A borate buffer was used at pH 8.5; ensuring that the reaction would occur. The reaction occurs instantaneously. The extract can be dried down and stored for several weeks with no apparent loss. The fluorescamine derivatized slaframine (slaframine-F) was resuspended in acetonitrile/water (35/65, v/v) and this, too, was stable over time, as seen in Figure 7. The HPLC analysis of the slaframine-F was first attempted using an Ultracarb 5 column (Phenomenex, Torrance, CA). This is a C-18 column especially designed to give better

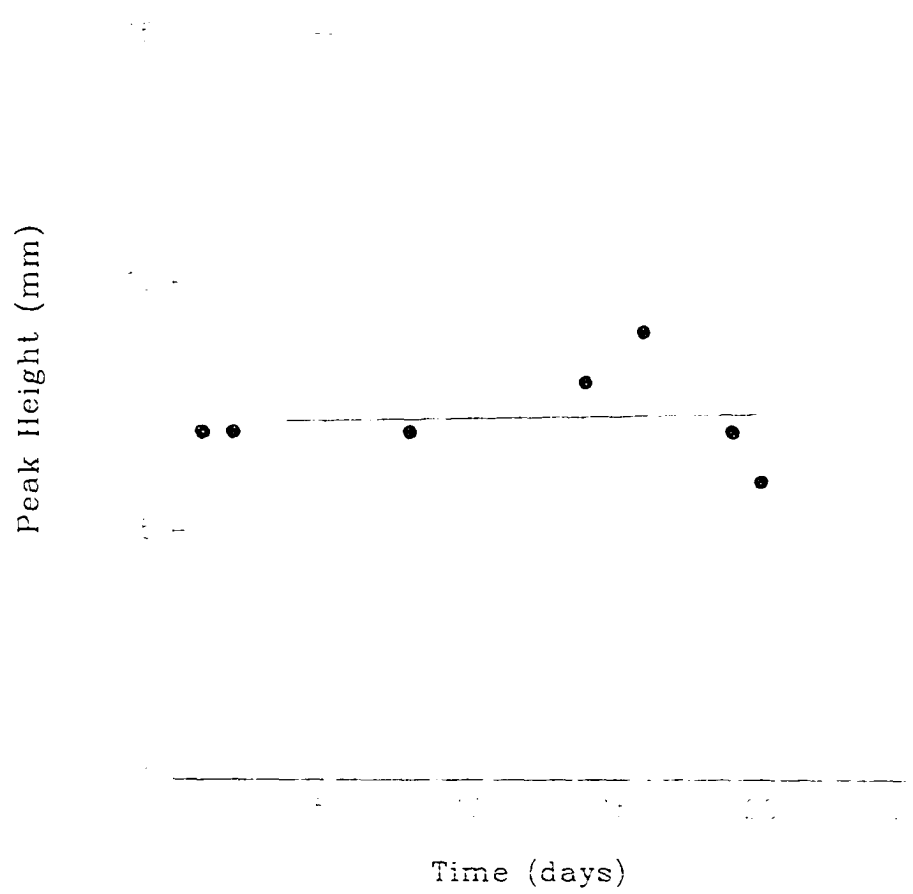


Figure 7. The stability of 1 ng slaframine-F for 20 days.

separation and sharper peaks for basic compounds by endcapping all of the active sites. The column worked well for some of the preliminary test samples and standards with an acetonitrile/water (35/65, v/v) mobile phase. However, when the large number of goat plasma samples were run, it was discovered that the retention time (R_t) of the analyte kept being reduced. This reduced R_t of slaframine moved until it overlapped other peaks. The forward shift in R_t caused the slaframine levels to appear increasingly higher. Many solvent systems were tried but, due to the limited pH range of this column (pH 2-8), were unsuccessful. A manual switching valve was installed to backflush the column between injections. This improved the situation, but it was not consistent (and it took a great deal of time). Therefore, it was decided that this column could not be feasibly used for this study. A β cyclodextrin column from Astec was tried, but it gave poor sensitivity. Several other columns, including the Perkin Elmer 3x3 C-18 column, were tried but gave poor resolution.

The Hamilton PRP-1 C-18 column, which was rejected early in the analysis because it gave relatively broad peaks, was tried again. This column has a polymer C-18 base with a pH range from 1 to 13. Various mobile phases were tried in an attempt to sharpen peak resolution. A mobile phase on the acid side, acetonitrile/25 mM potassium monophosphate 10 mM TEA pH 2 (45/55, v/v) was tried, but this eluted too many early compounds that interfered. The mobile phase was then switched to the basic side, acetonitrile/20 mM sodium borate 10 mM TEA pH 12 (35/65, v/v), which gave a baseline separation. At pH 12, slaframine-F was resolved from the earlier peaks. The retention

time of the slaframine-F in this mobile phase was approximately 7 minutes. A typical HPLC run was 15 minutes between injection of samples, so no overlapping occurs. Because of the retention-time characteristics and the quality of the peak resolution, the Hamilton PRP-1 column utilizing an autosampler was used for the vast number of analyses required for this study.

C. Method Evaluation

Slaframine was extracted from plasma as described in the methods section (J). The impurities were removed by making the plasma acidic and extracting it with methylene chloride. The slaframine was partitioned into the methylene chloride by first making the remaining aqueous extract basic at a pH of 10. At this stage, the sample was relatively clean, and the slaframine was derivatized with fluorescamine for HPLC analysis. Table 2 shows a series of ten uncontaminated plasma samples spiked at a 0.1 ppm slaframine level for method evaluation. The mean recovery of the slaframine from plasma was 95% with a standard deviation of 8.16.

The procedure for extracting slaframine from plasma was also tried on the milk samples. Unfortunately, extracting milk with methylene chloride resulted in emulsion problems. This caused low and variable recoveries of the slaframine from milk. Therefore a C-18 Sep-Pak® cartridge procedure was developed for slaframine in milk. The milk was passed through the Sep-Pak® cartridge; collecting the slaframine onto the C-18 support. The slaframine was eluted with methanol/water (75/25, v/v). A salt

Table 2. Slaframine recoveries from plasma method evaluation (samples spiked at 0.1 ppm slaframine)

Spiked sample	% recovery ^a
1	90
2	104
3	104
4	85
5	85
6	103
7	102
8	90
9	88
10	100

^aMean recovery is 95% with a standard deviation of 8.

solution plus water was added to the eluted solvent. The pH of this solution was adjusted to 10, and the slaframine was extracted into the methylene chloride; eliminating the emulsion problem. Table 3 shows the results of five uncontaminated milk samples spiked at 50 ppb slaframine. The mean recovery of slaframine from milk was 91% with a standard deviation of 9.

The procedure for extraction of slaframine from saliva was similar to that used for plasma except that a saturated sodium chloride solution was added before the basic extraction step. The added salt forces the analyte into the methylene chloride fraction; increasing the slaframine recovery. Table 4 shows the recoveries from ten samples of saliva spiked at 0.1 ppm slaframine. The mean recovery was 82% with a standard

Table 3. Slaframine recoveries from milk method evaluation
(samples spiked at 0.05 ppm slaframine)

Spiked sample	% recovery
1	100
2	82
3	103
4	83
5	88

^aMean recovery is 91% with a standard deviation of 9.

deviation of 11. The slaframine recoveries in saliva were lower and more variable than in plasma or milk. Perhaps this was due to glycoproteins (mucins), which are known to be present in saliva (57). Slaframine being a charged molecule may be binding to these mucins resulting in lowered recoveries.

D. Dosing of Goats

In reviewing the literature, no work had been done using an IV dose of slaframine in goats. Most previous work used oral or IP dosing of slaframine. There were only two reports of using goats as experimental subjects dosed with slaframine. In those experiments, Aust (33,39) reported IP dosing goats at a 0.1 or 0.2 mg /kg levels of slaframine. In spite of this, goats remained the preferred subject animals for this experiment, and IV dosing remained the preferred method. Goats were preferred because they are ruminants, dairy animals, and easily available; as well as being of manageable

Table 4. Slaframine recoveries from saliva method evaluation (samples spiked at 0.1 ppm slaframine)

Spiked sample	% recovery ^a
1	71
2	77
3	79
4	99
5	71
6	73
7	93
8	84
9	97
10	71

^aMean recovery is 82% with a standard deviation of 11.

size. An IV dosing was used to obtain pharmacokinetic parameters from slaframine blood level curves. Aust (33) reported the successful IV dosing of a cat at a 0.3 mg/kg level of slaframine. With this information, it was decided to begin the pharmacokinetic study by dosing goats at a 0.3 mg/kg level of slaframine.

The first Saanen goat, number 8909, was randomly selected from the herd. The animal was weighed, placed in a separate pen, and provided with hay and water. The animal weighed 50 kg. To obtain a 0.3 mg/kg dose, 15 mg (0.3 mg/kg x 50 kg) of slaframine was needed. The dose was prepared as described in methods section (F). The next morning the IV catheter was placed in the left jugular vein. A urinary catheter was inserted into the bladder (with some difficulty) and urine was drained from the bladder.

Milk was stripped before the dose was given.

Goat 8909 was given an IV dose of 0.3 mg slaframine/kg body weight. Salivation began within 2.5-5 minutes of the dosing. Blood and milk samples were taken at times defined in the methods sections (I and J). Bloating of the goat was noticed at the 2-hour time period. The goat was removed from the stanchion several times for walks. At the 4.5-hour time period a stomach tube was inserted to relieve the bloating. The goat died at the 6-hour time period.

The goat was brought to the Iowa State University Veterinary Diagnostic Laboratory where a post mortem was performed by Dr. Carson. No excess fluid was observed in the lungs. Histopathology of the lung and the liver showed congestion. The kidney and heart were normal by histopathology. The cause of the bloating was not clear. Perhaps the combined effects of eating green hay, lack of mobility, and slaframine influenced the death of the goat.

The experimental plan was modified in two ways to avoid further goat mortality. First, the IV dose was reduced to 0.05 mg slaframine/kg of body weight. Second, the animals were fasted for 15 hours prior to dosing with slaframine.

Five goats were then randomly selected from the herd. On the day before dosing, subject goats were placed into separate pens and given water but no hay. On the day of dosing, the IV catheter was inserted into the left jugular vein. The milk was stripped before the dose was given to avoid dilution. No urinary catheter insertion was done as the previous goat, 8909, had failed to urinate. Each of the five Saanen goats were given a

bolus IV dose of 0.05 mg slaframine/kg body weight. Table 5 shows weights of the goats and the amount of slaframine used. The following calculation was used to determine the amount (in mg) of slaframine needed for each goat:

$$0.05 \text{ mg} \times \text{weight of goat in kg} = \text{mg of slaframine needed}$$

After dosing, samples of blood, milk, and saliva were collected as defined in the method sections (I, J, and K).

Table 5. Weights of goats and mg of slaframine used in pharmacokinetic study at 0.05 mg/kg dosage level

Goat #	Weight (kg)	Slaframine (mg)
8656	72	3.6
9106	38	1.9
9119	50	2.5
9122	45	2.25
9135	38	1.9

E. Summary of Goat Physiological Signs

Salivation started within 2.5 to 5 minutes after the goats were injected with the IV bolus dose of slaframine . Within about 5 to 10 minutes, the goats were observed to be quivering. Within 30 to 40 minutes, the goats appeared depressed and lethargic and their ears drooped downward. Between 30 minutes and one hour, profuse salivation became a steady drool. The goats coughed and tried to swallow their saliva. The goats' eyes became glazed, and the goats staggered; appearing to be off-balance. Between 1 to 2

hours lacrimation occurred in some of the goats. By the 2 hour time period the salivation slowed to a steady drip, but the goats still appeared depressed and lethargic. By the 3 hour time period salivation decreased to a slow drip. At this time some goats urinated and/or defecated, but, in contrast to the existing literature, no diarrhea was observed. The goats regained an interest in drinking and eating, breaking a period of anorexia similar to what has commonly been reported in the existing literature. By the 4-hour time period salivation stopped altogether or slowed to a negligible rate. By the 5-hour time period the goats appeared alert and well, completely recovered.

F. Saliva Results

Table 6 shows the volumes of saliva collected for each goat at various time periods after the IV dose was given. The time periods for collecting saliva varied greatly because this was not major emphasis of the research and required the manual and time consuming effort of holding a container to the goats mouth. The amount of salivation was greatest between 2 and 4 hours after dosing (Figure 8). The large deviations reflect goat variability and the irregular collecting periods. All samples were analyzed by HPLC and compared to a duplicate spiked sample. Slaframine was not detected in any of the saliva samples collected from the 5 goats. The lack of slaframine in saliva could be due to several factors: the level being far too low for detection, the slaframine being destroyed by freezing (this would appear to be highly unlikely), or that no slaframine entered the saliva.

Table 6. Volume (ml) of saliva collected after dosing goats with slaframine

Goat #	Time (hours)							
	0.5	0.75	1.0	1.5	2.0	3.0	4.0	5.0
8656		40.0	60.0	47.0	24.0	21.0		
9106	6.5		11.0			45.0		8.0
9119	12.0		16.5	1.5				
9122			4.0	21.5		110.0	49.0	
9135		18.0			77.0	35.0		11.0

G. Plasma Results

Plasma samples were collected as stated in the methods section (I) and analyzed as described in methods section (L) for the amount of slaframine in each sample. The extraction of the plasma samples was duplicated, and the average of the two determinations for each goat are listed in Table 7.

In the plasma of four of the five goats no slaframine was found in samples taken beyond the three hour point. Slaframine in the plasma of the fourth goat, goat 8656, was present up to the six hour time period. This goat was four years older than all of the other goats and this may have affected retention time.

A graph of the data from Table 7 is presented in Figure 9 on a semi-log scale with a regression line fit to the sample data means and dotted lines representing the 95% confidence interval around the regression. Table 8 lists the average level of slaframine in ppb vs. time in hours. This data is graphed in Figure 10 with a regression line and 95%

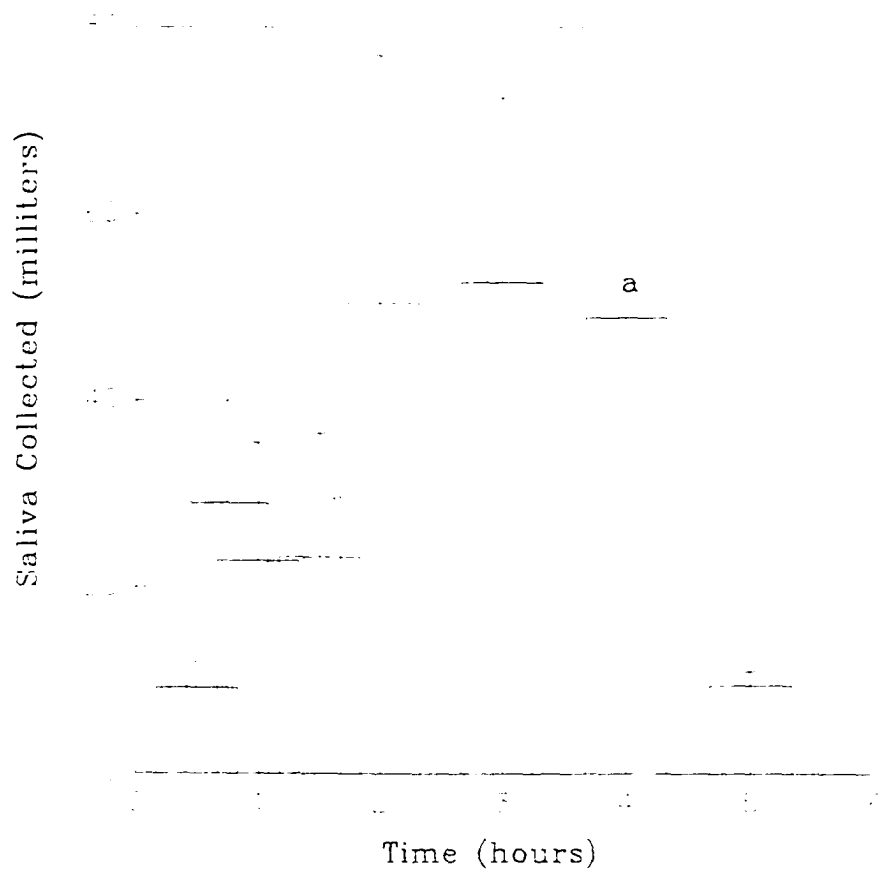


Figure 8. The average amount of saliva collected (ml) vs. time (hrs.) after a 0.05 mg / kg IV dose of slaframine using five goats.
a-Data point one goat only.

Table 7. Slaframine levels (ppb) in plasma samples from goats after 0.05mg/kg IV dose of slaframine

Time (hours)	Goat #				
	8656 ^a	9106	9119	9122	9135
0.042		24.4	11.8	24.0	64.2
0.083	38.0	15.3	33.3	16.0	45.8
0.166	21.5	8.3	24.0	14.0	18.7
0.25	15.0	6.3	14.7	7.4	14.2
0.5	9.5	3.7	8.0	5.3	9.5
0.75	10.2	2.9	6.9	3.4	6.7
1.0	8.6	1.7	5.9	2.2	4.1
1.5	7.8	1.1	3.1	1.1	2.1
2.0	6.0	0.4	2.8	0.8	1.6
3.0	5.1	0.0	2.5	0.6	0.9
4.0	3.7	0.0	0.0	0.0	0.0
5.0	1.7	0.0	0.0	0.0	0.0
6.0	2.2				
7.0	0.0				
8.0	0.0				

^aSamples were analyzed to 48 hours for goat #8656. No slaframine was detected beyond 6.0 hours.

confidence intervals. Graphs are shown in Figure 11 with the same data and regression.

but show the standard error bars instead of the confidence intervals.

The sample data listed in Table 7 was analyzed with PCNONLIN 4.2, a pharmacokinetics program, as discussed in the methods section (O). The data was analyzed using one-, two-, and three-compartment models. To determine which of these models provided the best fit for the data, the individual data points were analyzed with

each of the models. The output obtained from each model was then evaluated with respect to the correlation coefficient (y , \hat{y}), the degrees of freedom (df), the AIC criteria, the standard error of the estimated parameters, and a series of graphs.

The AIC criteria equals $(-2) \log(\text{maximum likelihood}) + 2 (\# \text{ of parameters})$. This is a Bayesian mathematical analysis to predict model fitting. The lower the AIC criteria the better the fit (58).

The standard errors of the estimated parameters were given a rating of good (G), fair (F), or poor (P) based on their t values. Parameters that resulted in t values with probabilities that were less than 0.05 were rated good. Parameters that resulted in t values with probabilities that were greater than 0.05 were rated poor. Parameters with t values falling in between these ranges were rated fair.

A series of four graphs (identified as graphs 1 through 4) generated by each model were used to evaluate the fit of data to the model. Graph 1, x vs. observed y and calculated y , was generally rated good when the majority of predicted and observed values are equal or close to equal. Graph 2, observed y vs. weighted calculated y , was rated good when a straight line was formed. Graph 3, weighted calculated y vs. weighted residuals, and graph 4, x vs. the weighted residual, y , should fall close to zero (the ideal case) or have close to as many points above and below the zero line to be rated good. Graphs which clearly do not meet these criteria are rated poor. Graphs which fall in between good and poor were rated fair. Some examples of these graphs and their ratings can be seen in Appendix A.

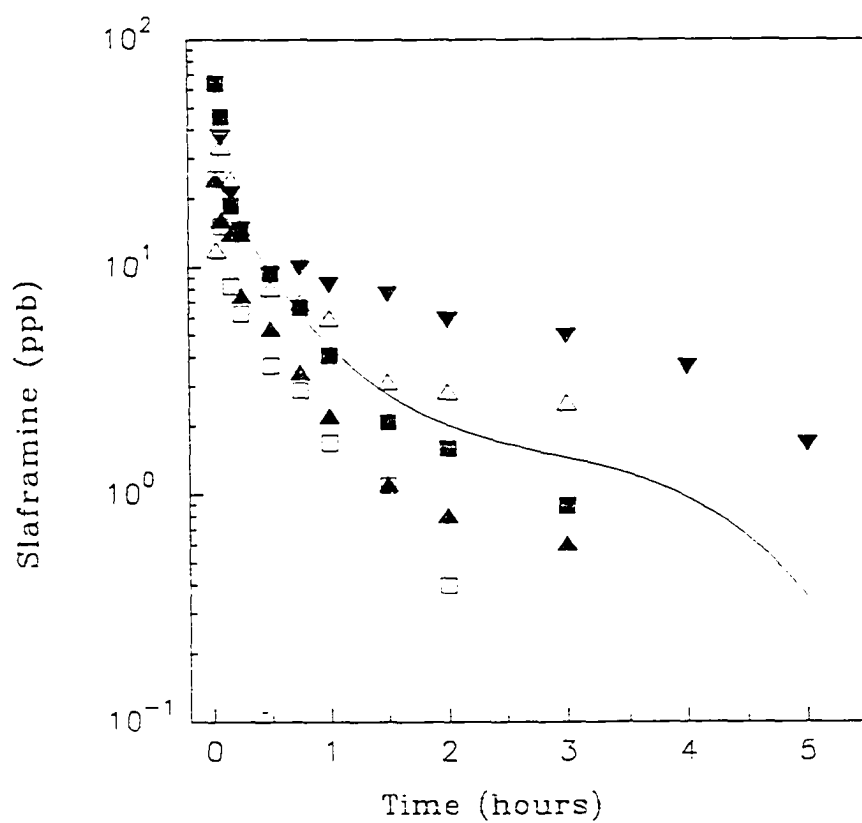


Figure 9. The amount of slaframine (ppb) vs.time (hrs.) from plasma after a 0.05 mg/kg slaframine IV dose on a semi-log scale showing data points from all five goats.

Table 8. Average slaframine levels (ppb) in plasma samples from five goats

Time (hours)	Slaframine (ppb)
0.042	31.1
0.083	29.7
0.166	17.3
0.25	11.5
0.5	7.2
0.75	6.0
1.0	4.5
1.5	3.0
2.0	2.3
3.0	1.8
4.0	0.7
5.0	0.4

The results of these rankings for the plasma data from each goat fitted to each of the three models are shown in Tables 9-12. In each of these tables, good-fair-poor rankings for graph 1 are replaced by actual counts of the data sample points where the observed y equalled the predicted y . Table 9 shows the data for each goat fitted to a one compartment model. Only goats 9106 and 9122 show some fit to the one compartment model; having good correlation values with 10 df, fairly low AIC values, and fair-to-good standard errors. However, most graphs for these goats rated poor and less than half of the predicted values in graph 1 matched the observed values. Thus, the one-compartment model did not appear to adequately fit the observed data on the rate of slaframine elimination from goat plasma.

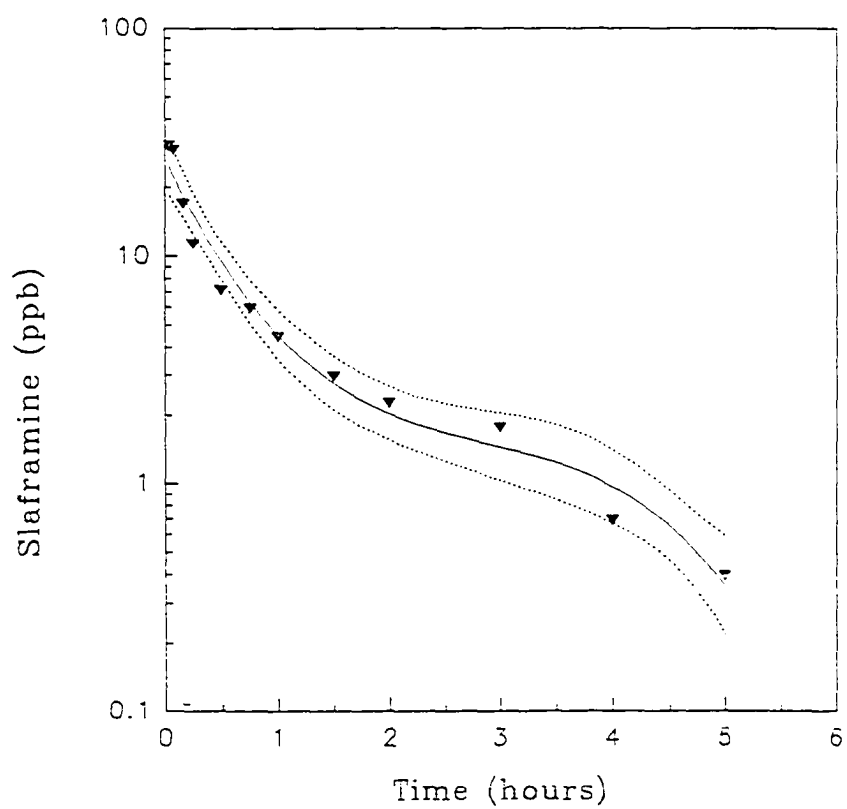


Figure 10. The average amount of slaframine (ppb) vs. time (hrs.) from plasma after a 0.05 mg/kg slaframine IV dose on a semi-log scale showing 95% confidence intervals.

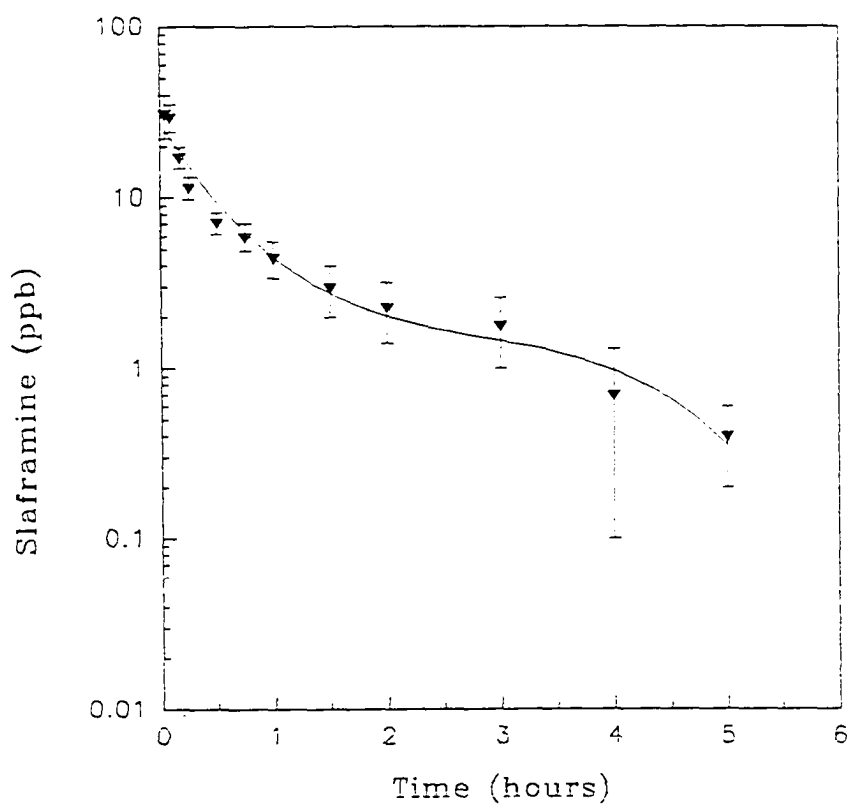


Figure 11. The average amount of slaframine (ppb) vs. time (hrs.) from plasma after a 0.05mg/kg slaframine IV dose on a semi-log scale showing standard error bars.

Table 9. One-compartment model data summary for slaframine clearance using PCNONLIN

Criteria	Goat #				
	8656	9106	9119	9122	9135
y, yhat	0.907	0.984	0.854	0.981	0.989
df	9	10	10	10	10
AIC	65.17989	43.88782	72.91577	44.26696	64.66489
Graph 1	1	2	3	4	6
Graph 2	P	P	P	P	P
Graph 3	P	P	P	P	P
Graph 4	P	P	F	P	P
Std. error	F	F	F	G	G

Table 10 shows the evaluation criteria for data fit in a three compartment model. Only the data from goats 9106 and 9122 could be successfully run through the PCNONLIN 4.2 analysis program using this model. The plasma data from the other three goats resulted in an "ill conditioned program error," for the three compartment model. In goat 9122 the correlation, df, AIC, and the first graph were all good. However, the majority of graphs were poor, indicating errors with respect to residuals. This probably accounts for the very poor standard errors of the estimated parameters in this model; some being upwards of 10 times the value of the actual parameters.

Tables 11 and 12 show the individual goat data run through both of the two-compartment models available in PCNONLIN 4.2. The values from both models are favorable for all goats with exception of goat 9119. For this goat the AIC was high, the

Table 10. Three-compartment model data summary for slaframine clearance using PCNONLIN

Criteria	Goat #				
	8656 ^a	9106	9119 ^a	9122	9135 ^a
y, yhat		1.00		0.994	
df		6		6	
AIC		-8.72663		36.38457	
Graph 1		8		8	
Graph 2		G		P	
Graph 3		P		P	
Graph 4		P		F	
Std. error		P		P	

^aData not suitable for estimation due to poor fit within model parameters.

Table 11. Two-compartment model data summary for slaframine clearance using PCNONLIN model 7

Criteria	Goat #				
	8656	9106	9119	9122	9135
y, yhat	0.999	1.00	0.855	0.992	0.998
df	7	8	8	8	8
AIC	18.51331	-10.83994	76.79773	37.02444	44.69210
Graph 1	7	8	3	6	7
Graph 2	G	G	P	G	G
Graph 3	F	F	G	F	F
Graph 4	F	F	G	G	G
Std. error	G	G	P	F	G

Table 12. Two-compartment model data summary for slaframine clearance using PCNONLIN model 8

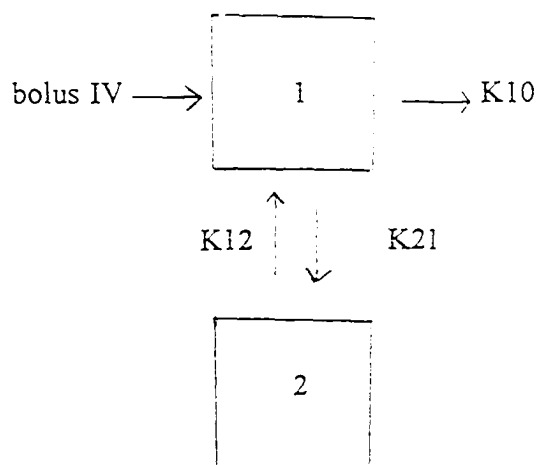
Criteria	Goat #				
	8656	9106	9119	9122	9135
y, yhat	0.999	1.00	0.855	0.992	0.998
df	7	8	8	8	8
AIC	18.53302	-10.84033	76.77143	37.02137	44.69503
Graph 1	7	8	3	6	7
Graph 2	G	G	P	G	G
Graph 3	F	G	G	G	G
Graph 4	F	G	G	G	G
Std. error	G	G	F	F	G

standard error was poor, the matching of actual and predicted values in graph 1 was low, and graph 2, observed y vs. weighted calculated y, did not form a straight line. For the other four goats, however, the best fit was obtained with the two-compartment model. Furthermore, a better fit was obtained from the two-compartment model using macroconstants as the primary parameters (Model 8) relative to the two-compartment microconstant model (Model 7). The differences between these two models was most apparent in the evaluation of the output graphs, as summarized in Tables 11 and 12.

The mean value for each timed sample across all five goats (listed in Table 8) was analyzed using PCNONLIN 4.2, Model 8, the two compartment model with IV bolus input and first order output using macroconstants as the primary parameters, where:

$$C(T) = A e^{-\alpha t} + B e^{-\beta t}$$

and



Model 8 was used for this analysis, because β , the overall elimination rate constant, is the most important pharmacokinetic parameter, and it is one of the primary parameters in Model 8 (59).

Table 13 shows the estimated parameters from model 8 for the mean of all five goats. Looking at the α rate constants, which represent the distribution diffusion phase from the central (1) to the peripheral (2) (tissue compartment), goat 9119 varied quite significantly from the rest of the animals. This could be responsible for the inherently high standard error for goat 9119 shown in Table 13. Also, the half life for the α phase was the longest for goat 9119 at 22 minutes. The β half life for the elimination phase of slaframine shows a variance from 25 minutes to 2.25 hours with a mean of 1.28 hours. The elimination phase of slaframine in goats 9106, 9135, and 9122 was very short ranging

Table 13. Estimated parameters from plasma data using PCNONLIN model 8

Parameters	Goat #					
	8656	9106	9119	9122	9135	Mean
A [ng/ml]	68.920420 (4.621549)	35.053434 (0.895421)	22.370246 (27.163673)	20.92704 (4.134611)	90.405224 (5.474531)	33.847595 (2.924315)
α [h ⁻¹]	11.544948 (0.805527)	17.898215 (0.777168)	1.879270 (3.045749)	12.407924 (6.439807)	13.057667 (1.750109)	6.714806 (1.299314)
B [ng/ml]	11.914159 (0.617016)	8.371511 (0.386537)	2.830716 (29.267920)	11.731859 (4.750630)	13.582750 (3.745858)	7.514404 (2.591182)
β [h ⁻¹]	0.313183 (0.032881)	1.502172 (0.082925)	0.308044 (3.274642)	1.626413 (0.626643)	1.059072 (0.361336)	0.539733 (0.260393)
$t_{1/2\alpha}$ [h ⁻¹]	0.060039 (0.004185)	0.038727 (0.001680)	0.368838 (0.597183)	0.055863 (0.028964)	0.053084 (0.007108)	0.103227 (0.019954)
$t_{1/2\beta}$ [h ⁻¹]	2.213232 (0.232135)	0.461430 (0.025447)	2.250156 (23.896250)	0.426181 (0.164040)	0.654485 (0.223075)	1.284242 (0.618962)
AUC [ng/ml/h]	44.011880 (2.741900)	7.531426 (0.161138)	21.093012 (40.159614)	8.899570 (1.158145)	19.748680 (2.270073)	18.963199 (3.797239)
Cl _b [ml/kg/h]	1.136057 (0.070879)	6.638849 (0.142271)	2.370453 (4.521102)	5.618249 (0.732941)	2.531815 (0.291482)	2.636686 (0.528849)
MRT [h]	2.771668 (0.306959)	0.507121 (0.026914)	1.714569 (14.468242)	0.513622 (0.162595)	0.640044 (0.215127)	1.399859 (0.672889)
Vd [ml/kg]	3.6	4.4	7.7	3.5	2.4	4.9

Parenthesis denote standard errors of the parameters.

from 25 to 39 minutes; however, the elimination phase of slaframine in goats 8656 and 9119 was longer at approximately 2.2 hours. This could be due to variation of the rate of metabolism within in the species.

The volume of distribution (V_d), the sum of volumes of the central and peripheral distribution compartments, can be calculated from the data in Table 13 using the following formula (59):

$$V_d = \frac{\text{dose}}{(AUC)(\beta)}$$

While experiments on the protein binding of slaframine have not been done, low V_d values indicate that xenobiotics are highly protein-bound in blood (59). The V_d value for slaframine was 4.9 ml/kg, which suggests it is highly protein-bound in goat's blood.

Body clearance (Cl_b) is a measure of the ability of a substance to be removed by the organs of elimination (the sum of all clearance processes in the body) (59). From Table 13 we can see that the mean clearance value for slaframine was 2.6 ml/kg/h. This indicates a rapid elimination.

H. Milk Results

Milk samples were collected as stated in the methods section (J). Table 14 shows the volumes of milk collected after dosing the goats with slaframine. The average amount of milk collected at each collection time is graphed in Figure 12. The five goats used in

this experiment were near the end of their lactation stage, and this may have contributed to the lack of milk from goat 9122. Slaframine may also shut down milk production in the lactating goats. Slaframine is known to depress milk production: as reported in the literature by Aust et al. (11) and Crump et al. (60). Contributing to this loss of milk production was the loss of fluid by 1) the increase of salivation and 2) the depression of the animal, which resulted in a lack of water intake. Thus, with the loss of fluid from the goat and lack of replenishing fluid to the goat, milk production suffers with a decrease in

Table 14. Volume (ml) of milk collected after dosing goats with slaframine

Time (hours)	Goat #				
	8656	9106	9119	9122	9135
0.0	60.0	122.0	740.0	332.0	164.0
0.25		29.0	68.0	45.0	25.0
0.5	17.0	12.0	17.0	4.0	10.0
0.75			3.5	1.0	
1.0	49.0	2.5	2.5	0.5	1.5
1.5	29.0	4.5	1.5	0.4	2.5
2.0	15.0	1.5	1.7	0.0	1.2
3.0	7.5	4.5	1.5	0.0	1.5
4.0	19.0	3.5	0.0	0.0	1.5
5.0	32.0	2.5	0.5	0.0	1.0
6.0	9.0				
7.0	6.0				
8.0	13.0				
24.0	575.0	62.0	5.0	2.0	10.5
31.5	400.0				
48.0	945.0				

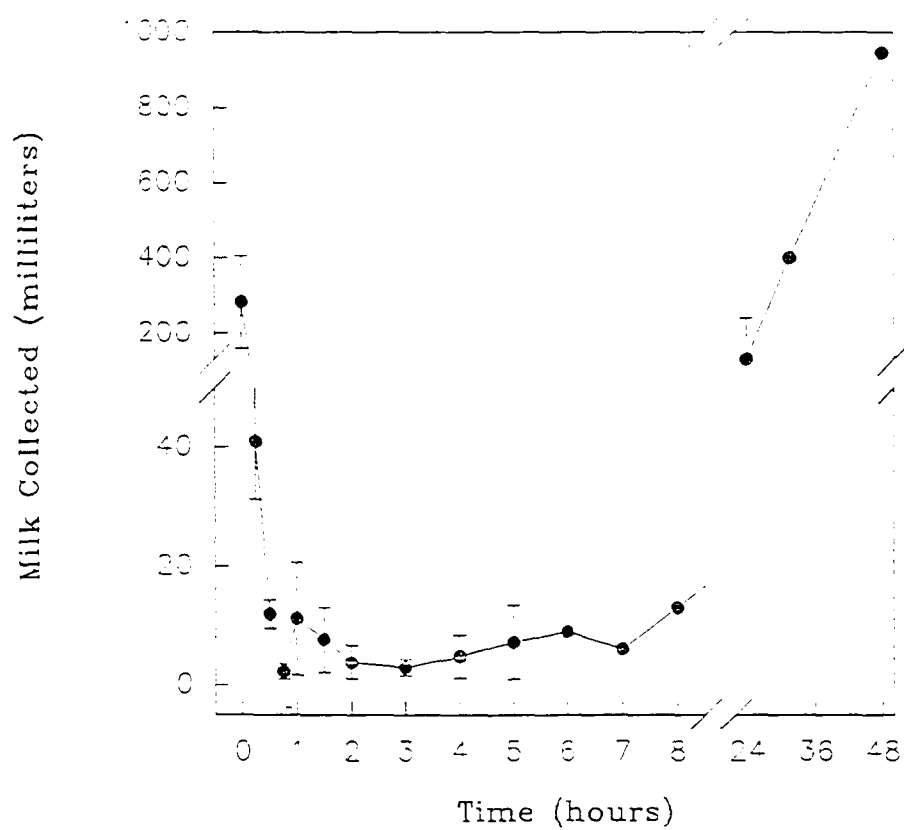


Figure 12. The average amount of milk collected (ml) vs. time (hrs.) after an IV dose of 0.05 mg/kg slaframine.

output. Whether slaframine is affecting milk production at the hormonal level is not known.

Table 15 shows the level of slaframine detected in each of the milk samples taken. Slaframine was found to be transferred into the milk and was seen in the earliest samples taken (zerotime plus fifteen minutes). Figure 13 illustrates this graphically by showing the average amount of slaframine transferred into the milk over time. It was expected that slaframine levels in the milk would initially increase during an absorption phase and then would steadily decrease as a result of the repeated stripping of milk from the udder (61).

Table 15. Slaframine levels (ppb) in milk samples from goats after 0.05mg/kg IV dose of slaframine

Time (hours)	Goat #				
	8656	9106	9119	9122	9135
0.25		0.66	3.2	3.5	0.47
0.5	15.9	0.72	3.7	2.4	0.77
0.75			4.7	3.9	
1.0	19.0	2.1	5.7	7.6	7.2
1.5	17.5	1.7	9.0	9.5	2.0
2.0	14.1	2.8	8.6		1.1
3.0	4.4	1.0	9.1		0.4
4.0	9.4	1.3			0.35
5.0	4.9	1.1	15.1		0.0
6.0	5.2				
7.0	2.1				
8.0	2.3				
24.0	2.0	0.9	7.7	25.9	0.8
31.5	4.0				
48.0	1.5				

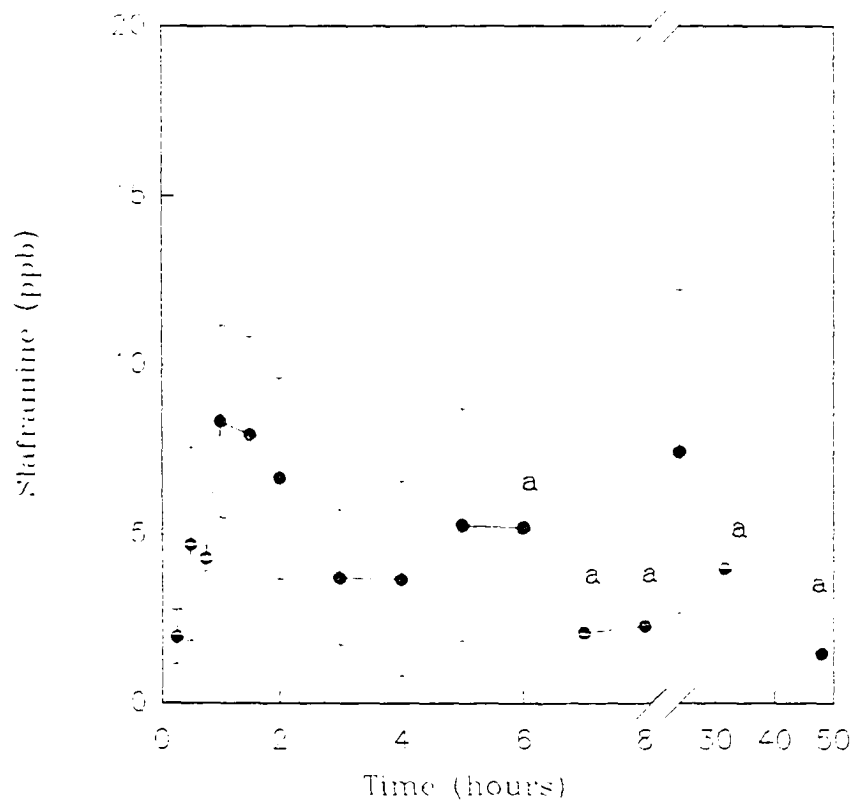


Figure 13. The average amount of slaframine (ppb) vs. time (hrs.) transferred into milk from a 0.05 mg / kg IV dose of slaframine using five goats.
a-Data points from goat 8656 only.

The levels of slaframine seem to increase, fall off, increase, and fall off again during the first eight-hour period. Perhaps this could be release from some other peripheral tissue (a deep compartment). Between the strippings at 8 and 24 hours the slaframine levels increased a second time. This, again, might be due to a peripheral release, or it might be due to slaframine concentration in the milk over this first extended (over 1 hour) time period between milk strippings. The table in Appendix B shows the total amount of slaframine in milk along with the dose given to each goat. The goats were given milligram levels but microgram level of slaframine was seen in the milk, a factor of 1000 difference.

Milk samples were collected to the 48 hour time period for only the first animal, goat 8656. For the remaining goats milk samples were collected only up to the 24 hour time period. This change was made because slaframine levels in the plasma samples (which were analyzed immediately) of goat 8656 were observed to be zero after 6 hours. This led to the assumption that slaframine would be eliminated from the milk within 24 hours of dosing. In retrospect, the analysis of milk samples (which were done as a group after all goats had been dosed and sampled) indicated that slaframine was still present in the milk of goat 8656 at the 48 hour sampling period. A longer sampling time period would be needed to fully understand the elimination of slaframine from the milk.

Due to the truncated sample data, it was difficult to utilize the milk data in a pharmacokinetic model. A preliminary estimation of the model could be approximated by using the data from goat 8656. In a one-compartment model, drugs or toxins in milk have

only one elimination constant. However, in a two- or a three-compartment model, the drug or toxin that is eliminated into the milk compartment can diffuse back to the central compartment (unlike the situation that exists in the case of urinary excretion). As a result of this complication, another elimination constant (a K_{20} in a two compartment model or a K_{30} in a three compartment model) must also be interpreted (62). This results in what is referred to as a diffusion model. The diffusion model takes into account the protein-bound and the unbound toxin with the unionized toxin migration in both directions across the mammary membrane (61,63,64).

None of the models supplied by PCNONLIN 4.2 incorporates secondary elimination, so using the program for this analysis was not feasible. However, it was possible to use the milk-to-plasma ratio (M/P) and to compare the result obtained here to the resultant graphs for one-, two-, or three-compartment models based on Wilson et al. (62). Figure 14 shows the M/P vs. time for goat 8656. Appendix C shows Wilson's M/P vs. time graphs for one-, two-, and three-compartment models. A comparison indicated that a two-compartment or a three-compartment model was most consistent with the milk data collected from goat 8656.

Figure 15 shows slaframine levels in milk and plasma samples from goat 8656 graphed vs. time on a semi-log scale. The data from milk parallels the plasma quite well, given the observation that the milk contains a higher level of slaframine. This can be explained by the pH partition theory using the Henderson-Hasselbalch equation (65) for basic molecules:

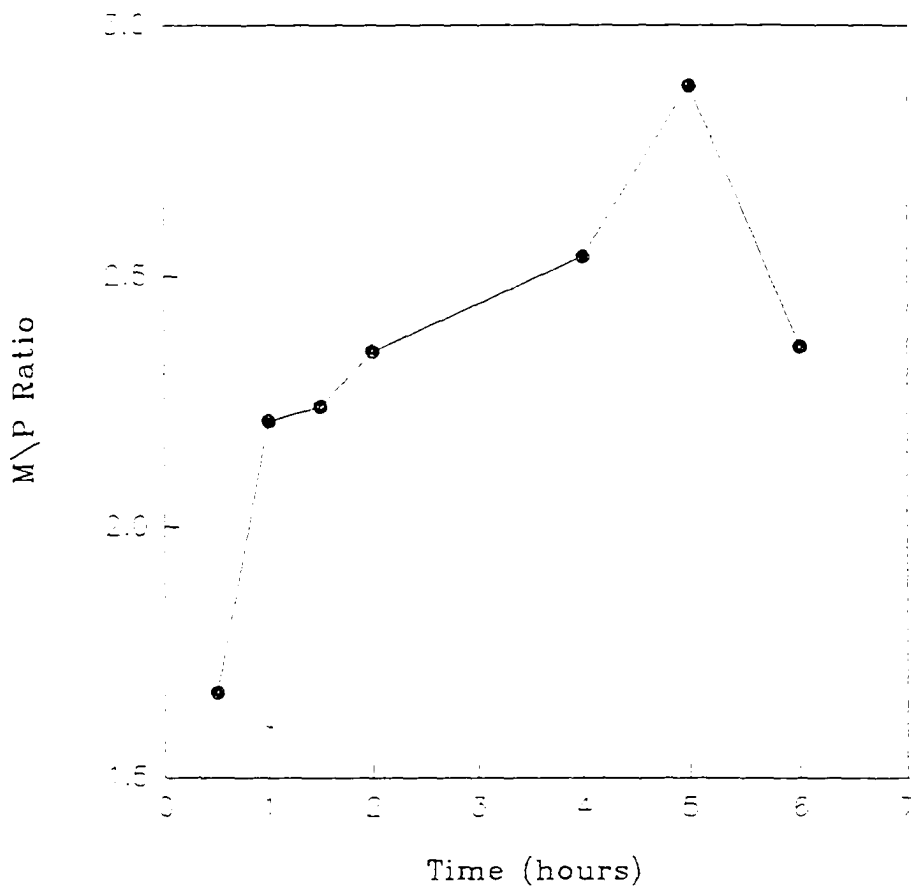


Figure 14. Milk / Plasma (M/P) ratio vs. time (hrs.) from goat 8656 after a 0.05 mg/kg IV dose of siaframine.

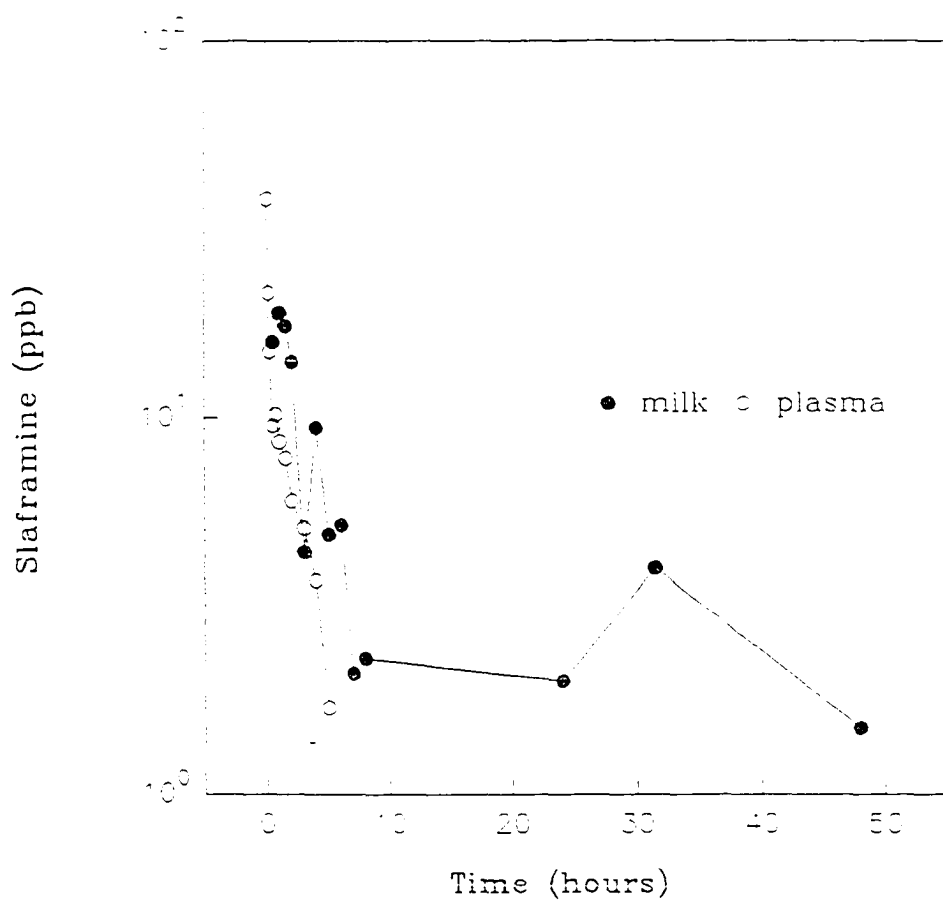


Figure 15. Slaframine (ppb) vs. time (hrs.) from goat 8656 in milk and plasma after a 0.05 mg/kg IV dose of slaframine on a semi-log scale.

$$\text{pH} - \text{pK}_a = \log \frac{\text{unionized}}{\text{ionized}} = \log \frac{U}{I}$$

Where: slaframine $\text{pK}_a = 9.1$ (66)

plasma $\text{pH} = 8.0$ (obtained experimentally)

milk $\text{pH} = 6.7$ (obtained experimentally).

In plasma for goat 8656:

$$8.0 - 9.1 = \log \frac{U}{I}$$

$$-1.1 = \log \frac{U}{I}$$

$$0.08 = \frac{U}{I}$$

If $U=1$, then:

$$0.08 = \frac{1}{I}$$

$$I = \frac{1}{0.08} = 12.5$$

$$U + I = 13.5$$

In milk for goat 8656:

$$6.7 - 9.1 = \log \frac{U}{I}$$

$$-2.4 = \log \frac{U}{I}$$

$$0.004 = \frac{U}{I}$$

If $U=1$, then:

$$0.004 = \frac{1}{I}$$

$$I = \frac{1}{0.004} = 250$$

$$U + I = 251$$

Thus, the ratio between the plasma and the milk was determined to be approximately 1 to 250. The diffusion gradient favors the milk side so higher levels of slaframine are deposited into the milk as shown in figure 15.

Experiments using centrifugation ultrafiltrate devices were tried on a few plasma and milk samples to look at the protein-bound vs. the unbound toxin. But difficulty in reproducible spike recoveries and the total clogging of the membrane with the milk samples made this work unfeasible. Figure 16 shows milk data from goat 8656 (slaframine vs. time) on a semi-logarithmic scale. By using the slope (beta) of the lower

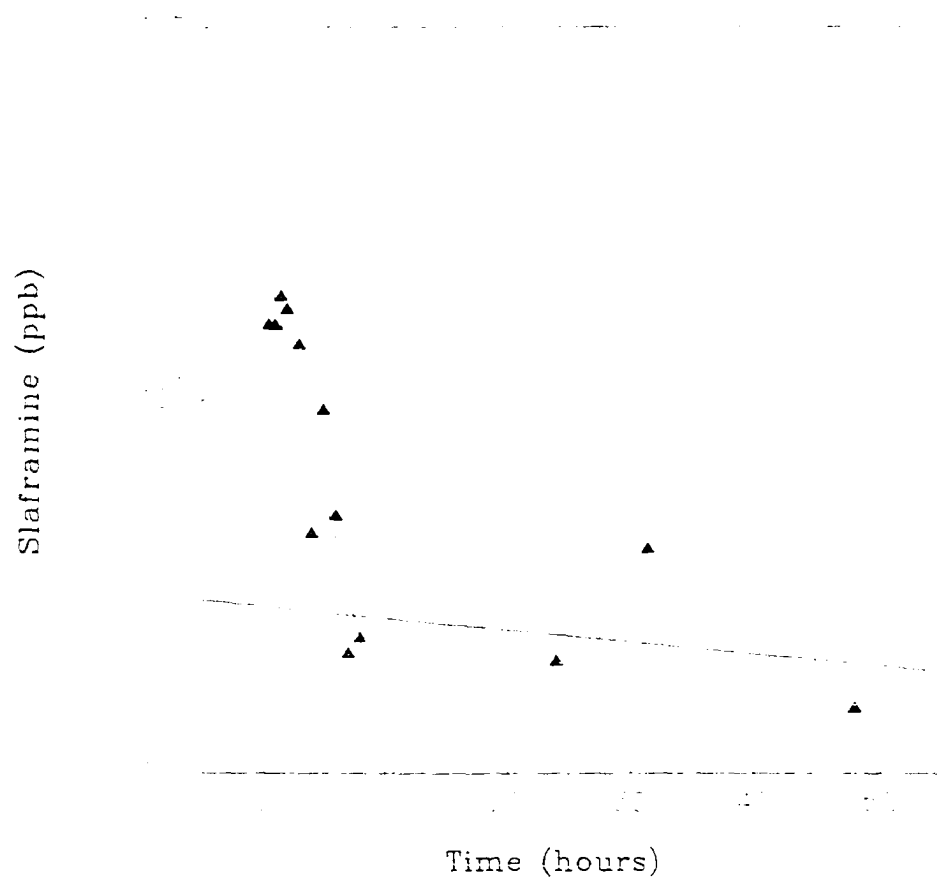


Figure 16. The average amount of slaframine (ppb) vs. time (hrs.) transferred into milk from 0.05 mg / kg IV dose of slaframine on a semi-log scale for goat 8656.

line of the elimination phase, we can calculate a rough estimate of the $t_{1/2}$ of the slaframine in milk using the formula:

$$t_{1/2} = \frac{0.693}{\beta}$$

$$t_{1/2} = \frac{0.693}{0.03} = 23 \text{ hours}$$

There exists a significant potential for error in this estimation as a result of using one animal at one dose level. The half life for milk should be equal to the half life of plasma. Several reasons may exist for the relatively long half life in the milk when compared to the plasma. Either the analytical method needs to be more sensitive in order to detect lower levels of slaframine in the plasma (a possibility in a three-compartment model), or a deep pharmacokinetic compartment may exist which may result in a later release of slaframine. The diffusion gradient contributes to both theories.

L. Suggested Future Experiments

While this research has conclusively shown that slaframine was transferred into milk, many questions have been left unanswered as a result of this research. Future experiments can help delineate some of these questions.

The bioavailability of slaframine in the milk can be determined if the AUC in milk is calculated. Then the percentage of the slaframine dose that is transferred to the milk

can be determined. The toxic risk remains unknown for the young goats, since slaframine must be metabolized into the 6-ketoimine to be activated. There is currently no data on how well slaframine is metabolized by young goats. There is also a potential risk that slaframine can enter the human food chain through dairy products. However, the stability of slaframine through the dairy processing system is also unknown. The potential exists for more experimental work to further define and quantify each of these risks, since the methodology for identifying and quantifying the presence of slaframine in milk now exists.

More research could further investigate elimination rates of slaframine in goats. Other IV dosing levels of slaframine should be tried to determine if the rate of elimination is dose dependent. A repeat of the current study's 0.05 mg/kg IV dose level of slaframine should be done with milk samples taken for a longer period of time (out to 2 or 3 half-lives) to more accurately track the final elimination of slaframine from the milk. Also other routes of excretion, such as urine and feces, could be investigated.

IV. SUMMARY

This research was undertaken to provide a preliminary study of the pharmacokinetics of slaframine in lactating goats. Particularly, it was designed to see if slaframine was transferred into milk. While substantial previous work had been done on the effects of slaframine intoxication on various species, no previous investigation has directly addressed the movement of slaframine through the body.

To complete this study a more sensitive method for slaframine analysis has been developed utilizing fluorescamine (fluorescent derivatizing agent) and HPLC. Methods for the extraction of slaframine from plasma, milk, and saliva have been developed. The method developed utilizing the fluorescamine derivatized slaframine gave a ten fold increase in detection levels over the previous GC technology. The slaframine fluorescamine derivative was stable for several weeks; allowing storage of samples between extraction and analysis time. The plasma and saliva procedures used a liquid/liquid partition between an acid/base partition into methylene chloride. The mean recovery for plasma using the methods developed was 95% with a standard deviation of 8 and, for saliva, was 82% with a standard deviation of 11. The milk procedure used a C-18 Sep-Pak® cartridge clean-up with a mean recovery of 91% with a standard deviation of 9.

The pharmacokinetic study of slaframine in lactating goats was initiated with an IV bolus dose of 0.05 mg slaframine/kg body weight. Analysis of the plasma data indicated

a two compartment model. The mean half-life for the elimination rate of slaframine was 1.28 hours. The V_d values indicated that slaframine was highly protein-bound in the goat blood. The CL for slaframine at this dose was rapid with a mean value of 2.6 ml/kg/h. The preliminary milk data from goat 8656 indicated a two or three compartment model. With a two compartment model a deep compartment may also exist due to the half-life for the elimination of slaframine in milk being 23 hours. A three compartment model could possibly be the case (with or without a deep compartment), but confirmation of this case would require that lower levels of slaframine in plasma would need to be detected. A higher level of slaframine was seen in milk than in plasma. This is consistent with the pH partition theory that indicates a theoretical M/P ratio of 20:1; using experimentally determined pH values for milk and plasma. When milligram levels of slaframine were given microgram level of slaframine was transferred into milk, a factor of 1000 difference.

Due to the ability of slaframine to be transported into milk a potential hazard exists for young nursing goats. More experimental work is needed in this area to determine the percentage of slaframine available to the young goat dependent on the mother's dose level and how young goats metabolize slaframine.

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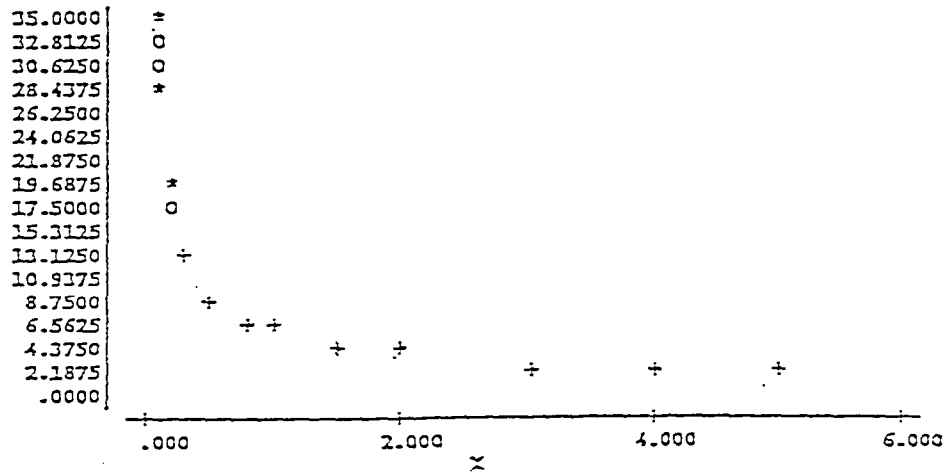
APPENDIX A

EXAMPLE GRAPHS FOR MODEL EVALUATION

Graphs rated good;

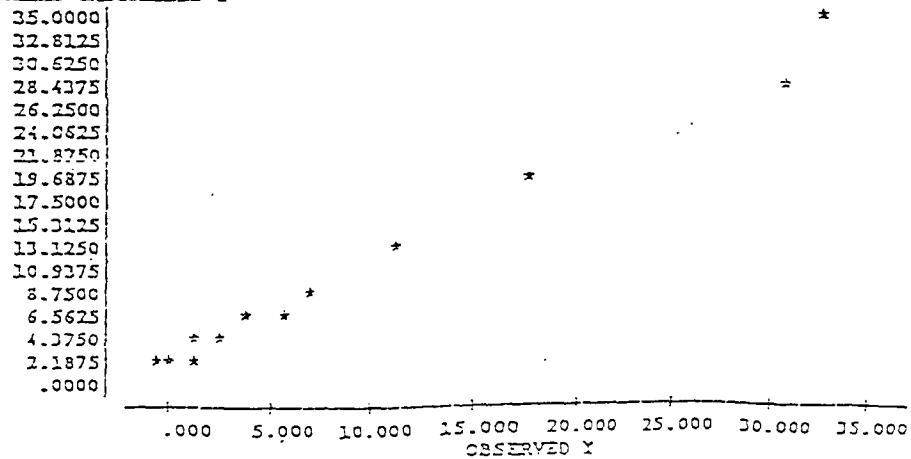
PLOT OF X VS. OBSERVED Y AND CALCULATED Y

Legend : * = predicted, O = observed, + = predicted & observed



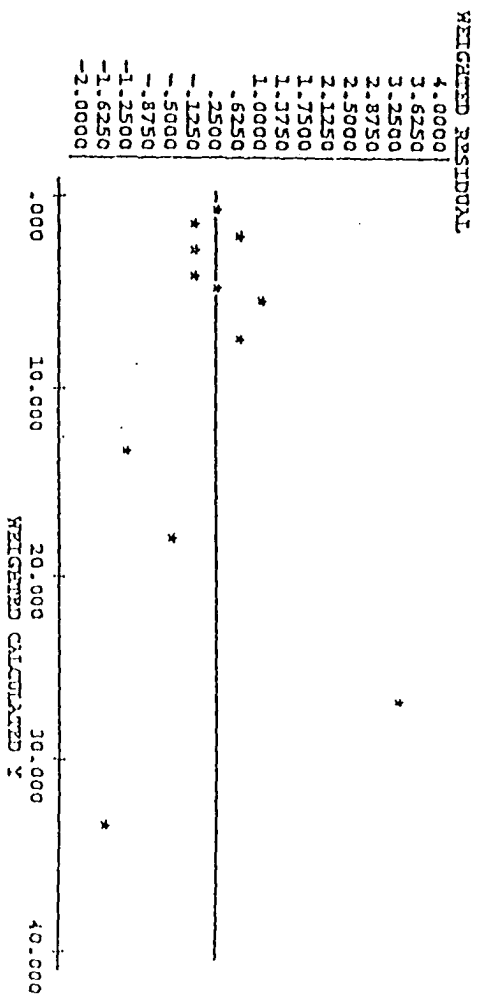
PLOT OF OBSERVED Y VS. WEIGHTED CALCULATED Y

WEIGHTED CALCULATED Y

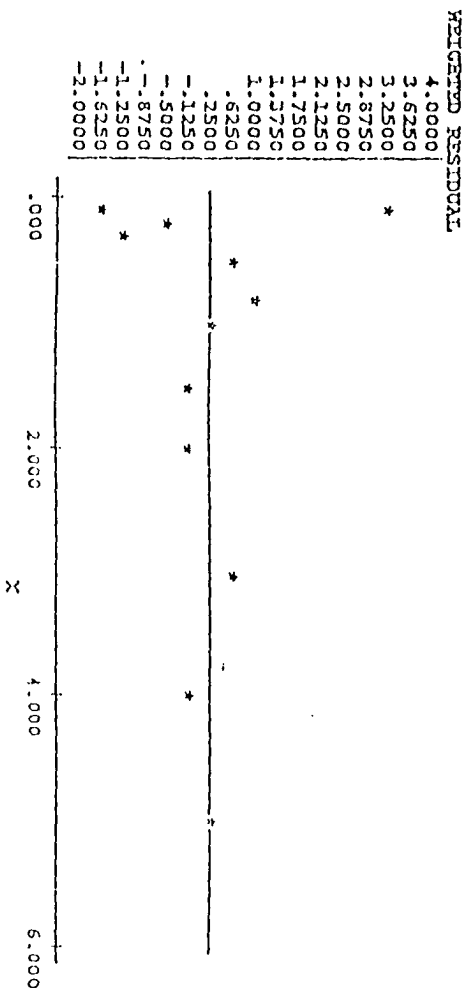


Graphs rated good:

PLOT OF WEIGHTED CALCULATED Y VS. WEIGHTED RESIDUAL

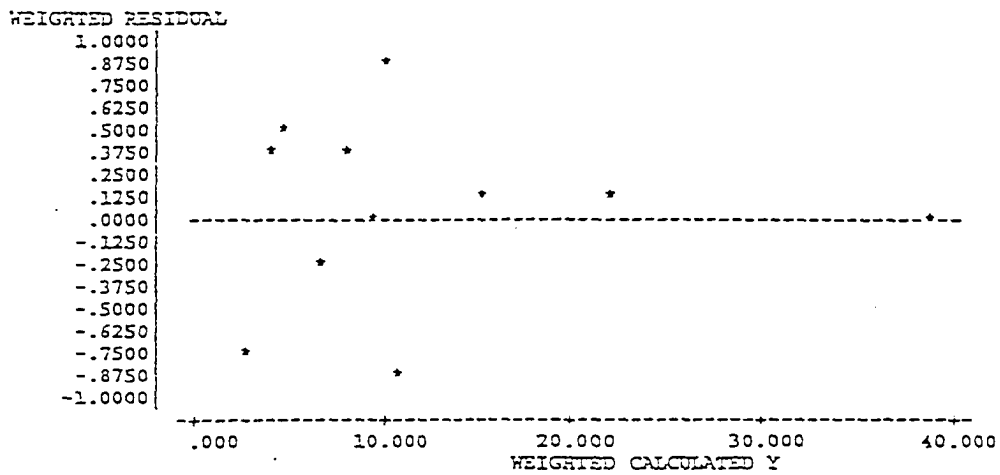


PLOT OF X VS WEIGHTED RESIDUAL Y

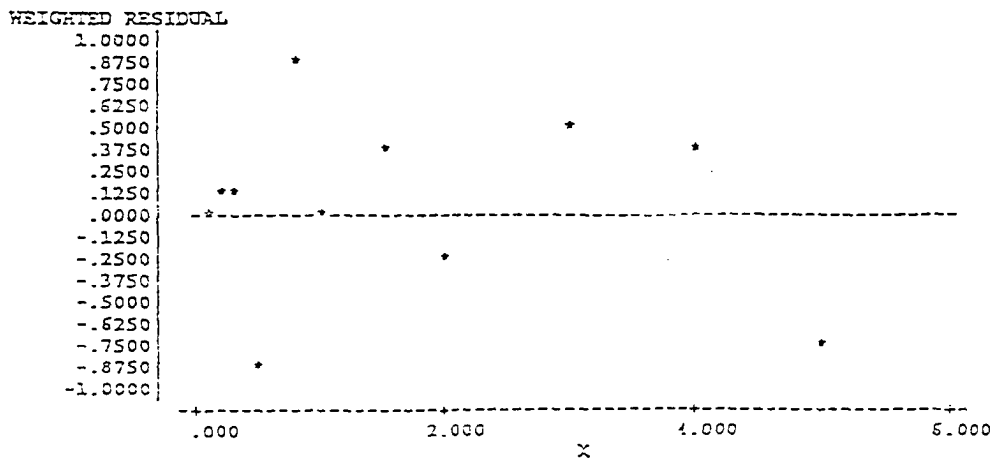


Graphs rated fair;

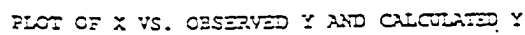
PLOT OF WEIGHTED CALCULATED Y VS. WEIGHTED RESIDUAL



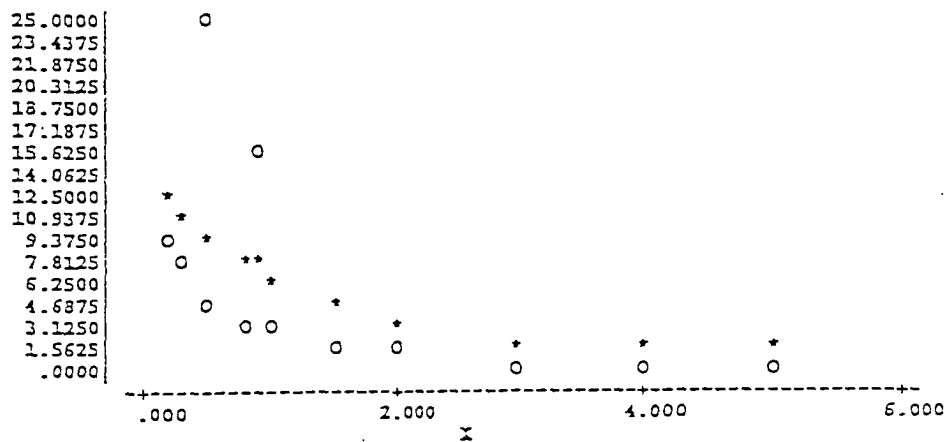
PLOT OF X VS WEIGHTED RESIDUAL Y



Graphs rated poor;

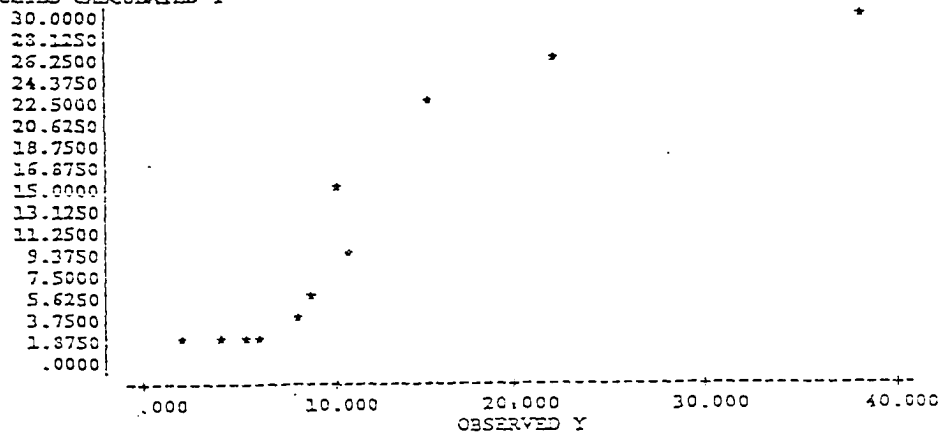


Legend : * = predicted, O = observed, + = predicted & observed



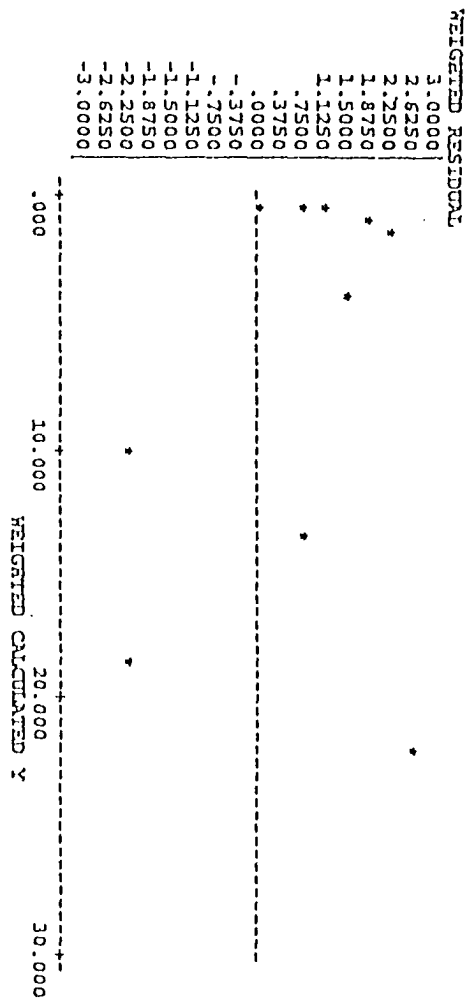
PLOT OF OBSERVED Y VS. WEIGHTED CALCULATED Y

WEIGHTED CALCULATED Y

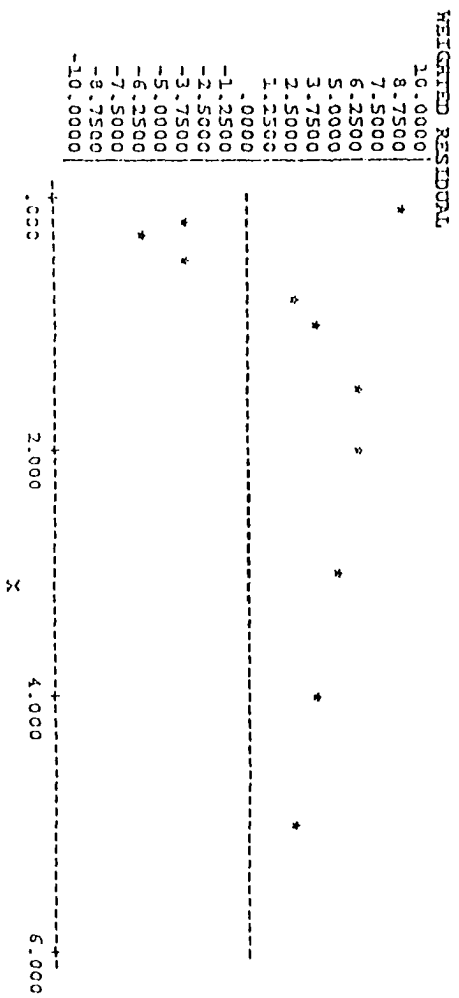


Graphs rated poor;

PLOT OF WEIGHTED CALCULATED \bar{y} VS. WEIGHTED RESIDUAL



PLOT OF \bar{x} VS WEIGHTED RESIDUAL \bar{y}



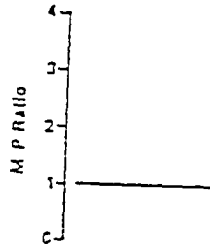
APPENDIX B**TOTAL AMOUNT OF SLAFRAMINE IN MILK AFTER DOSE**

Time (hours)	Goat #				
	8656	9106	9119	9122	9135
0	0	0	0	0	0
0.25		19.1	217.6	157.5	11.8
0.5	270	8.6	62.9	9.6	7.7
0.75			16.5	3.9	
1.0	931	5.3	14.3	3.8	10.8
1.5	507.5	7.7	10.5	3.8	5.0
2.0	211.5	4.2	14.6		1.3
3.0	33	4.5	13.7		0.6
4.0	178.6	3.9			0.5
5.0	156.8		7.6		
6.0	46.8				
7.0	10.5				
8.0	29.9				
24.0	1150	55.8	38.5	51.8	8.4
31.5	1600				
48.0	1417.5				
<hr/>					
Slaframine(ng) in milk	6543.1	109.1	396.2	230.4	46.1
<hr/>					
Slaframine(mg) dose given	3.6	1.9	2.5	2.25	1.9

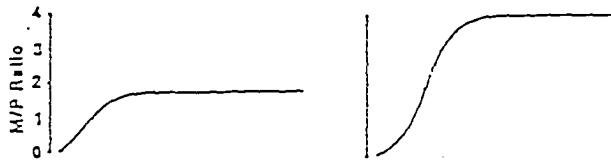
APPENDIX C

GRAPHS FOR MILK/PLASMA RATIO MODELS

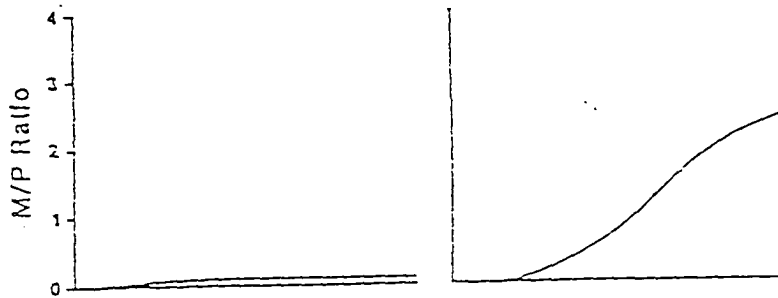
One Compartment Model



Two Compartment Model



Three Compartment Model



APPENDIX D**SUPPLY VENDORS FOR MATERIALS**

American Type Culture Collection
Rockville, MD
26280 *Rhizoctonia leguminicola* freeze dried culture

Becton Dickenson
Sandy, UT
14 gauge, 5 inch angiocatheter

Central Stores, Iowa State University
Ames, IA
Sylvania cool white bulbs

College of Veterinary Medicine clinic pharmacy, Iowa State University
Ames, IA
sterile heparin solution 10 units/ml

CoStar
Cambridge, MA
0.45 μ m sterile filter

Dairy Farm, Iowa State University
Ames, IA
5 - Saanen lactating does (goats)

Fisher Scientific Co.
Chicago, IL
soxhlet apparatus
methylene chloride
sodium chloride
sodium carbonate
1 liter, 125, 250 ml separatory funnels
Buchii Roto Evaporator
glacial acetic acid
HPLC grade methanol
HPLC grade chloroform
5, 10, 50, 100, 1000 ml beakers

10-20 ml septum vial
EDTA 10 ml vacutainer blood collection tubes
Falcon 2057 snap cap test tubes
ACS grade HCL
ACS grade sodium hydroxide
sodium sulfate
HPLC grade acetonitrile
TLC tank
250 ml erlenmeyer flask
pasteur pippettes
Optima grade acetone
sodium borate
Genie 2 vortex mixer
15 ml screw cap glass test tubes

Hamilton
Reno, NV
PRP-1 10 μ m 250 x 4.1 mm HPLC column
10 microliter syringe

IEC International Centrifuge
Needham Hts., MA
clinical centrifuge
centrifuge

J.T. Baker
Phillipsburg, NJ
iodine crystals

McPherson, Division of S.I. Corp.
Acton, MA
FL-749 HPLC fluorescence detector with HSA assembly

Midland Scientific Inc.
Omaha, NE
500 ml glass blender

Midwest Grain Products Co.
Weston, MO
95% ethanol

Millipore Corp.
Marlborough, MA
millipore Q water

Millipore - Waters Chromatography Division
Marlborough, MA
990 Diode Array detector
6000A HPLC pump
C-18 Sep-Paks®

Perkin Elmer Corp.
Norwalk, CT
3x3C pecoshere cartridge C-18 HPLC column

Rochester Scientific
Rochester, NY
2 dram glass vials

Statistical Consultants, Inc.
Lexington, KY
PCNONLIN 4.2

Sherwood Medical Inc.
St. Louis, MO
5, 12 ml monoject syringes
18 gauge 1.5 inch monoject needles

Sigma Chemical Co.
St. Louis, MO
fluorescamine
triethylamine

Shimadzu
Columbia, MD
Sil 9 HPLC autosampler
CR 501 integrator

VWR Scientific
Ontario, Canada
standard 100 x 15 mm sterile disposable petri plates

Whatman Inc.
Clifton, NJ
reverse phase C-18 TLC plates

APPENDIX E

LIST OF ABBREVIATIONS

ATCC - American Type Culture Collection

A - Intercept of monoexponential absorption slope with ordinate

AUC - Area under the curve.

α - alpha

B - Intercept of back-extrapolated monoexponential elimination slope with ordinate

β - Beta

°C - degrees celsius

C-18 - Carbon chain of 18 units

^{14}C - Carbon 14

Cl_b - Body clearance

cm - Centimeters

C(T) - Blood concentration at time T

df - *degrees of freedom*

FAD - Flavin adenine dinucleotide

FMN - Flavin mononucleotide

g - gram

GC - gas chromatography

GI - Gastrointestinal

GOT - Glutamic oxaloacetic transaminase

h - hour

HCl - Hydrochloric acid

HPLC - High pressure liquid chromatography

I - Ionized

IM - Intermuscular

IP - Interperitoneal

IV - Intervenous

K₁₀ - Overall elimination rate constant

K₁₂ - Distribution rate constant for transfer from central to peripheral compartment

K₂₁ - Distribution rate constant for transfer from peripheral to central compartment

kg - kilogram

l - liter

LDH - Lactate dehydrogenase

mg - milligram

ml - milliliter

mM - millimolar

M/P - Milk to plasma ratio

MSD - Minimum salivary dose

N - Normality

NADPH - Nicotine adenine diphosphate

NANA - N-acetylneuraminic acid

nm - Nanometer

NMR - Nuclear magnetic resonance

NaOH - Sodium hydroxide

PC - personal computer

pH - Measure of hydrogen ion concentration

pKa - Measurement where the ratio of unionized and ionized species are equal (half-way titration point)

ppb - Parts per billion

ppm - Parts per million

® - Registered trademark

RBC - Red blood cell

Rf - Reference value

rpm - Revolutions per minute

Rt - Retention time

SAB-DEX - Saboraud dextrose media

SC - Subcutaneous

SD - Standard deviation

Slaframine-F - Fluorescamine derivatized slaframine

t - time

$t_{1/2}$ - Half-life

TEA - Triethylamine

TLC - Thin layer chromatography

U - Unionized

μg - microgram

μl - microliter

μm - micrometer

UV - Ultraviolet

v - volume

Vd - Volume of distribution

vs. - verses

WBC - White blood cell

Δ - delta

% - percent