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Short communication

New, sensitive high-performance liquid chromatography method for the determination of slaframine in plasma and milk

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Abstract

Slaframine was derivatized precolumn with fluorescamine. The derivatized slaframine was chromatographed isocratically using HPLC on a Hamilton PRP-1 C_{18} polymeric column with fluorescence detection. By using fluorescent derivatization, sensitivity was increased 100-fold over previously reported GC methods. A liquid–liquid partition was used to extract slaframine from plasma with a 95% recovery and a CV% of 8.4. A solid-phase extraction was used to extract slaframine from milk with a 91% recovery and a CV% of 9.8.

Keywords: Milk; Plasma; Slaframine; Mycotoxins; Toxins; Amines

1. Introduction

Slaframine is an alkaloidal mycotoxin produced by *Rhizoctonia leguminicola*. Both ''Blackpatch disease'' on legumes and ''Slobber disease'' in livestock have been linked to the same source of mold, *Rhizoctonia leguminicola*. ''Blackpatch disease'' is named for the dark spots of fungal growth which infect the plant and ''Slobber disease'' results from ingestion of mold infected feedstuffs by livestock.

Occurrence of this mycotoxin has been widespread through the central, southeastern, and southwestern states of the USA when climatic conditions are right [1–4]. The mold flourishes in periods of wet weather, high humidity, and mild temperatures between 25–29°C [1,2]. Infection of plant materials, especially clover, is often mistaken as the ripening of the plant. As a result, animals are fed infected clover and exposed unknowingly [1,2]. The fungus can overwinter and has been known to last up to two years in infected seeds [1]. All isolates of the fungus are known to produce the toxin [1,2].

Analysis of slaframine to date have included paper, thin-layer, and gas chromatography. Aust et al. [5], Wylie and Morehouse [6] and Byers and Broquist [7,8] used paper chromatography and visualized the toxin by ninhydrin or Dragendorff's reagent. Byers and Broquist [7] and Stahr [9] used normal-phase thin-layer chromatography visualizing with either Dragendorff's or vanillin followed by sulfuric acid. Both paper and thin-layer chromatography were sensitive to the microgram level. Gas chromatography has been used with either OV-17, OV-1, or SE-30 columns with a flame ionization detector. Hagler et al. [10] used a trimethylsilyl derivative of slaframine for GC analysis. Stahr et al. [11] used an acetate derivative of slaframine for GC analysis with a nitrogen–phosphorus detector. With large samples and extensive cleanups these methods allowed sensitivity to a level of tenths of micrograms.

The procedure reported here is a new sensitive method for analyzing low levels of slaframine. This

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procedure was developed to obtain pharmacokinetic data on this toxin and to investigate whether slaframine was transferred into the milk of lactating animals. The latter could possibly result in secondary exposures of this toxin. Since slaframine is quite toxic to animals, low exposure levels result in very low levels within biological samples. Earlier work by this investigator using reversed-phase thin-layer chromatography showed that a fluorescamine derivative could be made to visualize the slaframine [12]. This knowledge led to the development of this new sensitive HPLC method for slaframine detection.

2. Experimental

2.1. Chemicals

The slaframine used in this study was prepared as previously described in the author's dissertation [13]. All solvents were HPLC or reagent grade. All water was prepared through a Millipore cartridge system.

2.2. Sample collection

Blood samples were collected from a 2-year-old Saanen goat using 10-ml EDTA vacutainer tubes and were immediately inverted several times after collection to avoid clotting. The blood samples were then refrigerated. After completion of sampling, the blood samples were centrifuged in a clinical centrifuge for 15–30 min, and the plasma was removed. Two-ml aliquots of plasma were placed into 10-ml test tubes (2057 Falcon), labeled and then frozen at −10°C.

One quart of milk was obtained from a 2-year-old lactating Saanen goat (1 quart=946.3 ml). The milk sample was thoroughly mixed, and 3–5 ml samples of milk were placed in 10-ml test tubes (2057 Falcon), labeled, and frozen at −l0°C. All samples were thawed before the extractions were performed.

2.3. Plasma extraction

Two 1-ml volumes of plasma were placed in separate 15-ml screw-cap glass test tubes. One plasma sample was spiked with 0.1 μg slaframine. A 2-ml volume of 2 *M* HCl was added to the plasma tubes, vortex mixed, and centrifuged at 800 *g* for 15 min. The top, aqueous, layers from each of the tubes were then removed and placed in labeled 10-ml beakers. A 3-ml volume of 1 *M* NaOH solution was then added to the beakers, and the pH was adjusted to 10.0, with 10% sodium carbonate. The contents of the 10-ml beakers were then extracted separately with 2 ml of methylene chloride and centrifuged at 800 *g* for 15 min. This extraction was repeated a second time. The bottom methylene chloride layers were removed, passed through a Pasteur pipette containing sodium sulfate, and collected into a 2-dram glass vial (1 dram=3.697 ml). The methylene chloride in the 2-dram vial was evaporated to dryness under a nitrogen stream with low heat (50°C).

2.4. Milk extraction

A C_{18} Sep-Pak cartridge was activated with 4 ml of methanol then 4 ml of water using a 5-ml disposable syringe. An aliquot of 2 ml of goat milk and 2 ml of water were passed through the activated C_{18} Sep-Pak cartridge and the effluent discarded. A 5-ml aliquot of water was then passed through the C_{18} Sep-Pak cartridge and this effluent was discarded. A 30-ml volume of methanol–water (75:25, v/v) was passed through the C_{18} Sep-Pak cartridge, and the effluent was collected into a 100-ml beaker. Then 25 ml of water and 3 ml of saturated sodium chloride solution were added to the beaker. The pH of this mixture was adjusted to 10.0 with 10% sodium carbonate and then the mixture was extracted with two 10-ml volumes of methylene chloride in a 125-ml separatory funnel. The methylene chloride fractions were combined into a 50-ml beaker. This volume was concentrated to approximately 4 ml by a nitrogen stream with low heat (50°C), then passed through a Pasteur pipette containing sodium sulfate, and collected into a 2-dram glass vial. The methylene chloride in the 2-dram vial was evaporated to dryness under a nitrogen stream with low heat (50°C). This was repeated for 0.1 μg slaframine added to 2 ml milk samples.

2.5. Derivatization

Preparation of fluorescamine derivatives was done by adding 100 μl of a 25 m*M* sodium borate–100

m*M* HCl buffer at pH 8.5 to a dried residue of standard, sample, or spiked sample and vortex mixed. Then 100 μl of fluorescamine–acetone (6 mg:25 ml, w/v) was added and vortex mixed. This mixture was concentrated to dryness under a nitrogen stream with low heat (50°C). The resultant fluorescamine derivatives were used for HPLC analysis.

2.6. HPLC specifications

A Hamilton PRP-1 reversed-phase 10 μm 250× 4.1 mm HPLC column with a mobile phase of acetonitrile–20 m*M* sodium borate containing 10 m*M* 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 set at 365 nm Hg line, and the emission used a CF 400 filter. A Waters 6000A HPLC pump with a Shimadzu Sil 9A autosampler was used. The data were collected by a Shimadzu CR501 Chromatopac integrator set with an attenuation of 5.0 and a speed of 2 mm/min.

2.7. Stability test

A series of 0.05-μg slaframine-F standards had been stored at −10°C in 500 μl of acetonitrile–water (35:65, v/v) over a period of 20 days. A 10-μl volume of each standard was injected to equal 1 ng of slaframine-F over the 20-day period. The peak heights of these standards were measured and graphed.

2.8. Quantitation of samples

A standard curve of slaframine was prepared by using 0.05, 0.1, and 0.2 μg of slaframine and following the derivatization procedure. All standards and samples were dissolved in 500 μl of acetonitrile–water (35:65, v/v) prior to HPLC, and 10 μl injections were made for sample determinations. Quantitation of samples were calculated using linear regression. Determination of percent recovery was calculated by using the calculated amount divided by the added amount and multiplying by 100. The coefficient of variation (CV%) was calculated for the recoveries of fortified or spiked samples [14].

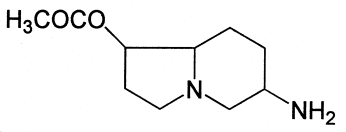

Fig. 1. Chemical structure of slaframine.

3. Results and discussion

The structure of slaframine contains a primary amine functional group at carbon-6 (Fig. 1). This allowed easy derivatization of slaframine by fluorescamine reagent. The derivative is formed in a basic environment between pH 8 and 9. A borate buffer was used at pH 8.5 to ensure that the reaction would occur. A series of the derivatized slaframine standards were run over a period of 20 days to test the stability of the derivatized slaframine in solution. The derivatized slaframine appears to be stable for several weeks, as shown graphically in Fig. 2. The fluorescamine derivatized slaframine (slaframine-F) gave a 100-fold increase in sensitivity over previous reported GC methods with a 0.1-ng standard giving a 75% full scale response. This procedure is also an advancement in that no method presently exists to analyze slaframine in plasma or milk.

The HPLC analysis of slaframine-F was first attempted using an Ultracarb 5 column (Phenom-

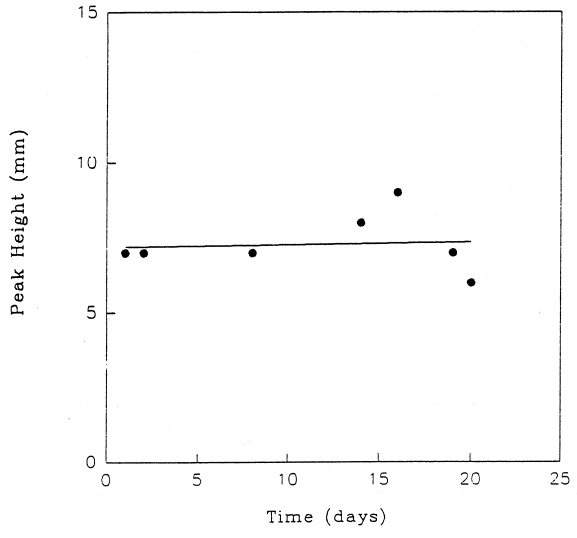

Fig. 2. Stability of fluorescamine derivatized slaframine over a time period of 20 days.

enex, Torrance, CA, USA). This column was said to be especially designed to give better separation and sharper peaks for basic compounds. Preliminary injections of standards worked well. However, after the injection of large numbers of goat plasma samples the retention time of slaframine-F became shorter with each additional injection. This indicated that other compounds from the plasma were collecting on the column; changing the column efficiency, and losing column resolution. Other solvent systems were tried, but due to the limited pH range (pH 2–8) they were unsuccessful. A manual switching valve was installed to backflush the column. This improved the procedure but was not consistent over time. A β-cyclodextrin column (Astec, Whippany, NJ, USA) and the 3×3 Pecosphere C_{18} column (Perkin-Elmer, Norwalk, CT, USA) were also tried but gave poor resolution. The Hamilton PRP-1, a polymer-based C_{18} column with a pH range of 1–13 was tried using various mobile phases. An acidic mobile phase, acetonitrile–25 m*M* potassium monophosphate, 10 mM triethylamine pH 2.0 (45:55, v/v) eluted too many early peaks which interfered. By using a basic mobile phase at a pH of 12.0, the slaframine-F was resolved from the earlier peaks. The retention time of the slaframine-F with a basic mobile phase was approximately 7 min. A typical HPLC run was 15 min between injections of samples. By using an autosampler, a vast number of samples can be run. Slaframine was extracted from ten fortified goat plasma samples as described in Section 2. The lipid and acidic impurities were eliminated by extraction with methylene chloride at a pH of 2.0. The slaframine was then partitioned into methylene chloride at a pH of 10.0. Fig. 3 shows a HPLC chromatographic run for a plasma sample. A series of results (Table 1) from the ten uncontaminated plasma samples, spiked at a level of 0.1 μg/ml slaframine for method evaluation, are shown. The mean recovery was 95% with a CV% of 8.4.

The procedure for extraction of slaframine from plasma was then tried on the milk samples. However, the extraction of the milk with methylene chloride resulted in an emulsion that made this procedure problematic by causing low and variable recoveries. The use of a C_{18} Sep-Pak cartridge was employed to eliminate the emulsion problems. The milk sample plus an equal volume of water were passed through an activated cartridge, collecting the slaframine on the cartridge. The slaframine was then eluted off the cartridge with 30 ml of 75% methanol. This solution along with added water and salt solution was ad-

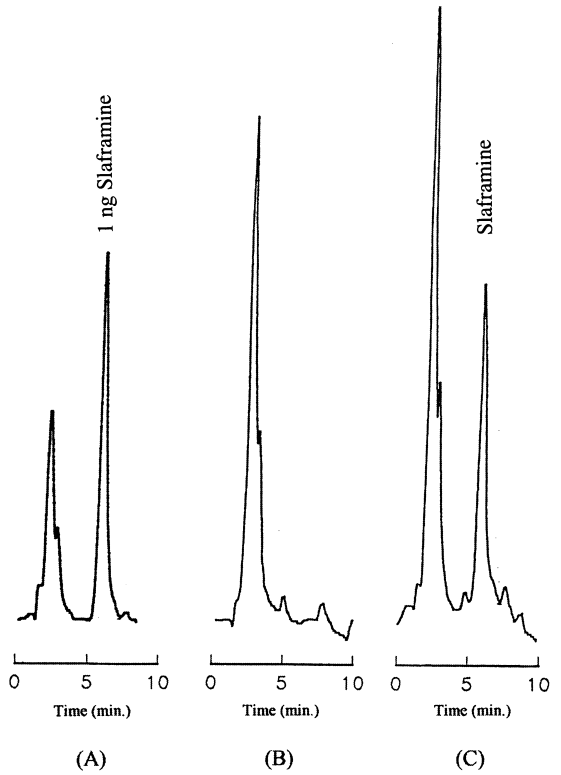

Fig. 3. A typical chromatogram for HPLC of spiked plasma sample showing (A) 1 ng slaframine standard, (B) uncontaminated plasma sample and (C) uncontaminated plasma sample spiked at 0.1 μg/ml.

Table 1
Recoveries of fluorescamine derivatized slaframine from plasma (samples spiked at 0.1 μg/ml slaframine)

Spiked plasma	Recovery (%)[a]
1	90
2	104
3	104
4	85
5	85
6	103
7	102
8	90
9	88
10	100

[a] Mean recovery is 95% with a CV% of 8.4.

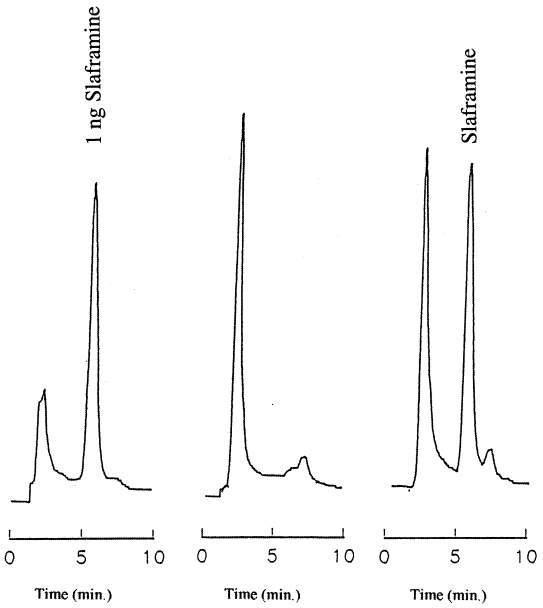

Fig. 4. A typical chromatogram for HPLC of spiked milk sample showing (A) 1 ng slaframine standard, (B) uncontaminated milk sample and (C) uncontaminated milk sample spiked at 0.05 μg/ml.

justed to a pH of 10.0, and then the slaframine was extracted into methylene chloride (as described in Section 2). Fig. 4 shows a HPLC chromatographic run for a milk sample. The results are shown (Table 2) for five uncontaminated goat milk samples spiked at 0.05 μg/ml slaframine for method evaluation. The mean recovery was 91% with a CV% of 9.8.

In conclusion, this HPLC method with precolumn fluorescamine derivatization provides a very sensitive and reliable assay for slaframine in both goat plasma and milk samples. Due to the low level of i.v. dosing of slaframine for a pharmacokinetic study, this HPLC procedure should facilitate the collection of data from lactating goats. Also, the stability of the derivatized slaframine should aid in the analysis of the vast number of samples which need to be analyzed.

Table 2
Recoveries of fluorescamine derivatized slaframine from milk (samples spiked at 0.05 μg/ml slaframine)

Spiked milk	Recovery (%)[a]
1	100
2	82
3	103
4	83
5	88

[a] Mean recovery is 91% with a CV% of 9.8.

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