Metabolism of [^{14}C]Flusilazole in the Goat

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[Phenyl(U)-^{14}C] and [triazole(3)-^{14}C]flusilazole ([(bis 4-fluorophenyl)]methyl(1H-1,2,4-triazole-1-ylmethyl)silane; **I**) were extensively metabolized when fed to lactating goats (*Capra hircus*). The primary metabolites identified in goat tissues and milk were bis(4-fluorophenyl)(methyl)silanol (**II**) and 1H-1,2,4-triazole (**III**). Concentrations of total radiolabeled residues in the milk ranged from 0.09 to 0.74 μg/mL. Concentrations of radiolabeled residues found in tissues when the [^{14}C] label was in the phenyl or triazole position, respectively, were 13.5 and 3.54 μg/g (liver), 8.74 and 0.75 μg/g (kidney), 0.41 and 0.52 μg/g (leg muscle), and 4.07 and 0.94 μg/g (back fat). Urine contained an additional major metabolite identified as [bis(4-fluorophenyl)](methyl)silylmethanol (**IV**) and its glucuronic acid conjugate (**V**). With either labeled form of flusilazole, the majority of the recovered radiolabel was excreted in urine or feces.

Keywords: *Fungicide; metabolism; goat; flusilazole; bis(4-fluorophenyl)(methyl)silanol; 1H-1,2,4-triazole; [bis(4-fluorophenyl)](methyl)silylmethanol*

INTRODUCTION

Flusilazole ([(bis 4-fluorophenyl)]methyl(1H-1,2,4-triazole-1-ylmethyl)silane, **I**), a member of the ergosterol synthesis inhibitor class of fungicides, is the active ingredient of NuStar fungicide. Flusilazole is useful in controlling a number of cereal diseases of wheat, and grapes. The mode of action of flusilazole (and other triazole- or other azole-containing fungicides) is by inhibition of the cytochrome P-450-dependent 14α-demethylation of lanosterol to form ergosterol, an essential component of fungal membranes (Henry and Sisler, 1984). Although there are upward of 20 related fungicides (containing triazole, imidazole, or other azole groups) for agricultural use, metabolism of this class of compounds in food-producing animals has not been extensively reported. As a part of the registration effort for flusilazole, the metabolism in lactating goats was determined, using both phenyl(U)-^{14}C- and triazole(3)-^{14}C-labeled forms of the molecule. The design of the study was such as to be in compliance with the registration guidelines of the U. S. Environmental Protection Agency (Schmitt, 1982). Thus, goats were dosed on successive days with [^{14}C]flusilazole, and the animals were sacrificed within 24 h of the final dose. Analysis of the excreta (urine) was conducted as necessary to identify metabolites. Once metabolites were identified, the nature of the residues in the edible tissues was determined by co-chromatography with authentic standards.

EXPERIMENTAL METHODS

Chemicals. Both [phenyl(U)-^{14}C]flusilazole (specific activity 21.1 μCi/mg, >99% radiochemical purity) and [triazole(3)-^{14}C]-flusilazole (specific activity 17.9 μCi/mg, radiochemical purity >99%) were obtained from DuPont NEN Products (Boston, MA). The specific activity was adjusted to a specific activity of either 1.06 μCi/mg (phenyl label) or 1.00 μCi/mg (triazole label) by addition of analytical standard grade flusilazole, prior to dosing. Unlabeled flusilazole and authentic reference standards used for chromatographic identification purposes were synthesized at DuPont Agricultural Products (Wilmington, DE). All solvents used for HPLC analysis were glass distilled (Baker). All other reagents and common chemicals were reagent grade or better. β-Glucuronidase (Type B1) was obtained from Sigma Chemical Co. (St. Louis, MO).

In-Life Phase. Lactating dairy goats were obtained by, and dosed at, Bio Enviro Test Laboratory (Bernville, PA). Goats were acclimatized for 9 days prior to the commencement of dosing. Before the start of dosing, the goat receiving [phenyl-(U)-^{14}C]flusilazole (hereafter referred to as the "phenyl goat") was placed in a steel metabolism cage and catheterized for urine collection; the goat receiving the [triazole(3)-^{14}C]flusilazole (hereafter referred to as the "triazole goat") was not catheterized. Throughout the dosing period, the goats were fed a commercially available dairy goat diet supplemented with alfalfa. On six (phenyl goat) or five (triazole goat) consecutive days, following the morning milking, the goats received 50 mg of either [phenyl(U)-^{14}C]flusilazole or [^{14}C-3-triazole]flusilazole. The test substance was placed into a size 000 capsule, which was in turn placed inside a larger gelatin capsule filled with goat chow to prevent crushing of the capsule during dose administration. This administered dose corresponded to a nominal dietary dose level of 50 μg/g, assuming 1 kg of daily total feed consumption.

Sample Collection. Milk was collected both in the morning and in the evening. After the first morning dose, the evening (p.m.) and the following morning (a.m.) milk samples were combined and treated as 1 day samples. Urine from the phenyl goat was collected through the catheter into polyethylene bottles kept in an ice bath. Urine from the triazole goat was collected in polyethylene bottles after passing through a feces-retaining screen. Urine samples were collected following the morning milking, and the total volume was determined daily. Fecal samples from the phenyl goat were collected on a metal tray located under a perforated metal sheet platform. Fecal samples from the triazole goat were collected on a mesh screen. The feces were collected each morning, weighed, and stored frozen at −20 °C. Animals were sacrificed by sodium pentathol injection approximately 10 h (phenyl goat) or 22 h (triazole) after administration of the final dose. After sacrifice, kidney, liver, muscle, and fat samples were taken and stored at −20 °C until analysis. No attempt was made to collect volatile metabolites from the goats.

Determination of Radioactivity in Tissues, Milk, Feces, and Urine. Six representative aliquots (0.2−1.2 g) were removed from the tissue and placed in paper combustion cones (Packard Instrument Co., Downers Grove, IL). Each combus-

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tion cone was placed in a glass scintillation vial, which was in turn covered with cheesecloth and placed in dry ice. After being frozen, the samples were lyophilized on a LabConco Model 8 Freeze Drier. These freeze dried tissues were combusted in a Packard Tri-carb sample oxidizer (Packard Instrument Co.). $^{14}CO_2$ released from the combustion was trapped in Oxisorb-CO_2TM (DuPont NEN Products) and mixed with Oxiprep-2 scintillation cocktail (DuPont NEN Products) before liquid scintillation counting (LSC) on a Tracor Model III liquid scintillation counter (TM Analytic). Fat samples were sequentially extracted with hexane, methylene chloride, and acetone before the solvent-extracted fat samples were analyzed for residual radioactivity by combustion analysis. The solvent extracts of the fat were combined, and the radioactivity contained therein was determined by LSC. Fecal samples were lyophilized prior to being finely minced in a Waring Blendor. Radioactivity in the feces was determined by combustion analysis. Aliquots of milk (1.0 mL) and urine (0.1 or 1.0 mL) were added directly to Formula-947 Liquid Scintillation Cocktail (DuPont NEN Products) before quantifying radioactivity by LSC.

Chromatography. *(A) Thin-layer chromatography* on silica gel plates (Merck, EM Scientific) was accomplished using the following systems.

TLC system 1 used toluene/ethyl ether/methanol (75/40/10, v/v/v).

TLC system 2 used acetonitrile/ethyl acetate/formic acid (175/50/1.5, v/v/v).

(B) HPLC analyses used the following systems.

HPLC system 1 consisted of 15 cm × 4.6 mm id Zorbax ODS HPLC column equipped with a 3 cm ODS guard column (Brownlee Labs). Chromatographic conditions were the following: mobile phase, acetonitrile/0.01 M NaH_2PO_4 (45/55, v/v); flow rate, 2 mL/min; column temperature, 45 °C.

HPLC system 2 consisted of 15 cm × 4.6 mm id Zorbax ODS HPLC column equipped with a 3 cm ODS guard column (Brownlee Labs). Chromatographic conditions were the following: mobile phase, acetonitrile/0.01 M NaH_2PO_4 (40/60, v/v); flow rate, 2 mL/min; column temperature, 45 °C.

HPLC system 3 consisted of 25 cm × 9.2 mm id Zorbax C8 semipreparative column. Chromatographic conditions were the following: mobile phase, acetonitrile/0.01 M NaH_2PO_4 (3/2, v/v); flow rate, 6 mL/min; column temperature, 30 °C.

HPLC system 4 consisted of 25 cm × 4.6 mm Zorbax SIL HPLC column. Chromatographic conditions were the following: linear mobile phase gradient from acetonitrile/methylene chloride/hexane/water (1/49.5/49.5/0.05, v/v/v/v) to (25/37.5/37.5/0.05, v/v/v/v) over 15 min; flow rate, 2 mL min; column temperature, 35 °C.

HPLC system 5 consisted of 25 cm × 9.2 mm Zorbax SIL HPLC column. Chromatographic conditions were the following: mobile phase, acetonitrile/methylene chloride/hexane/water (70/14.8/14.8/0.05, v/v/v/v); flow rate, 4 mL/min; column maintained at room temperature.

HPLC system 6 consisted of 25 cm × 4.6 mm Zorbax SIL HPLC column. Chromatographic conditions were the following: mobile phase, acetonitrile/methylene chloride/hexane/water (70/14.8/14.8/0.05, v/v/v/v); flow rate, 2 mL/min; column temperature, 35 °C.

Retention times and/or R_f values for authentic standards of flusilazole and putative metabolites are listed in Table 1.

Isolation and Purification of Metabolite II from the Phenyl Goat. An aliquot (100 mL) of the day 2 a.m. urine was diluted with an equal volume of methanol. The resultant precipitate was removed by centrifugation and discarded and the remaining supernatant extracted twice with hexane. These hexane extracts, which contained about 25% of the total radioactivity in the urine, were combined, reduced in volume through rotary evaporation at 40 °C, and applied to a silica gel thin-layer plate (EM Scientific). The plate was developed in TLC system 1.

Silica gel corresponding to the radiolabeled band at R_f = 0.7 [located using a TLC plate radioscanner (Berthold model LB 2832 linear analyzer)] was scraped from the plate, and the radioactivity was extracted from the silica with methanol.

Table 1. Chromatographic Behavior[a] of Flusilazole and Its Major Metabolites in the Goat

HPLC Retention Times (in Minutes)

standard	HPLC system[a] 1	2	4
flusilazole (**I**)	8.2	16.0	14.6
bis(4-fluoro-phenyl)(methyl)-silanol (**II**)	5.6	9.5	5.2
[[bis(4-fluorophenyl)-(methyl)silyl]-methanol] (**IV**)	6.3	10.8	6.6
oxy[bis[bis(4-fluorophenyl)-(methyll) (silane)]] (**VI**)	19.0	b	2.0

TLC R_f Values

standard	TLC system[c] 1	2
flusilazole (**I**)	0.30	0.32
bis(4-fluorophenyl)-(methyl)silanol (**II**)	0.75	*d*
1H-1,2,4-triazole**(III)**	0.06	0.20
[[bis(4-fluorophenyl)−(methyl)silyl]methanol] (**IV**)	0.63	*d*
V	origin	*d*
oxy[bis[bis(4-fluoro-phenyl)(methyl)(silane)]]**(VI)**	0.86	

[a] Descriptions of HPLC systems are in the text. Chromatographic behavior of standards in HPLC systems used only to isolate the metabolites have not been included in this table. [b] Not analyzed. [c] Descriptions of TLC systems are in the text. [d] Not analyzed.

After concentration of the methanol extract by rotary evaporation, the radiolabeled material was resuspended in acetonitrile/0.01 M NaH_2PO_4 (45/55, v/v) prior to further purification in HPLC system 1.

One minute fractions were collected, and aliquots of each were analyzed by LSC. The radioactivity eluting between 5 and 6 min was diluted with water to a final acetonitrile concentration of <10% and applied to a Bond-Elut C2 solid-phase extraction (SPE) column (Analytichem International). Radioactivity binding to the column was eluted with methanol, concentrated, redissolved in acetonitrile/0.01 M NaH_2PO_4 (40/60, v/v), and chromatographed in HPLC system 2. Purified Metabolite **II** eluted from the column with a retention time between 9 and 10 min, was isolated from the mobile phase as before by SPE, and was analyzed by mass spectrometry.

Isolation and Purification of Metabolite IV from the Phenyl Goat. Fifty milliliters of urine (day 1 p.m.) was applied to a 2 cm id by 3 cm (height) column filled with ethyl (C2) Sepralyte bulk chromatographic material (Analytichem International). After the urine was applied, the column was washed sequentially with the following: (1) 50 mL of 10% methanol in water (which was discarded); and (2) 50 mL of methanol. The methanol wash was dried under reduced pressure, resuspended in 1−2 mL of methanol, applied to a 1000 μm silica gel thin-layer plate (Analtech, Newark, DE), and developed in TLC system 1. After development, radioactivity was visualized on the plate as previously described.

Radioactivity that remained at the origin of the TLC plate (metabolite **V**) was extracted with a methanol wash. After rotary evaporation to remove solvent, the residue was redissolved in 5 mL of 0.01 M sodium phosphate buffer, pH 6.0. Approximately 10 mg of β-glucuronidase was added to this buffer solution and the mixture incubated overnight at 37 °C. Radioactivity in the reaction mixture was retained on a C2 Bond Elut SPE cartridge (Analytichem International) and eluted from the column with 5 mL of methanol. This methanol eluate was dried, applied to a silica gel TLC plate, and developed in TLC system 1. After development, the radioactivity present in the single radiolabeled band (R_f = 0.64; metabolite **IV**) detected by radioscanning was removed using previously described methods. Metabolite **IV** was further purified using HPLC system 3. Radioactivity eluting between 5.5 and 7.0 min was trapped, diluted 3-fold with water, and concentrated by SPE on a C2 Bond Elut. Purified metabolite **IV** was eluted from the Bond Elut with 27 mL of methanol

and the solvent removed by rotary evaporation. After isolation, metabolite **IV** was analyzed by mass spectrometry.

Isolation and Purification of Metabolite III from the Triazole Goat. An aliquot of urine (90 mL) collected on the second day of dosing was adjusted to pH 1.0 by the addition of 1.0 M HCl and applied to a column packed with Dowex 50 W−X8 strong cation-exchange resin (BioRad). Unretained impurities were washed from the column with 0.1 M HCl, while the majority of the radioactivity applied to the column was eluted with 2 M $(NH_4)_2CO_3$.

The alkaline wash was reduced to dryness by rotary evaporation, resulting in a large residue of solid material. Radioactivity was removed from these solids by repeated methanol washes. The combined washes were concentrated under a stream of nitrogen, applied as a band to a silica gel thin-layer plate, and developed in TLC system 2. Radioactivity associated with the band at R_f 0.31 (containing 92% of the radioactivity) was removed and redissolved in acetonitrile/methylene chloride/hexane/H_2O (70/14.8/14.8/0.05, v/v/v/v) and chromatographed in HPLC system 5. Radioactivity eluting between 17 and 25 min was collected, concentrated, and chromatographed in HPLC system 6. Radioactivity eluting between 9 and 10 min was collected and submitted for mass spectral analysis.

Mass Spectrometry. Metabolite **II** was analyzed by electron impact gas chromatography−mass spectroscopy using a Finnigan Model 4500 mass spectrometer. Gas chromatography utilized a 15 m J&W DB-1 fused silica capillary column with a 1 μm film and a helium gas flow of 1.5 mL/min. The column temperature program ran from 60 to 260 °C at a rate of 10 °C/min (injection port 225 °C). The source temperature of the mass spectrometer was 150 °C and the ionization energy 70 eV. Metabolite **II** was also derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), before analysis as described above.

Metabolite **IV** was analyzed by electron impact GC-MS using the instrument described above. Chromatography was conducted on a 15 M DB-1 fused silica capillary column containing a 0.25 μm film. The injection port temperature was at 225 °C, and the column was temperature programmed from 60 to 260 °C at 10 °C/min. A helium carrier gas was used at a flow rate of 1.5 mL/min. The source temperature of the mass spectrometer was 150 °C and the ionization energy 70 eV. A trimethylsilyl derivative of metabolite **IV** was prepared by reaction with BSTFA. This derivatized material was analyzed under the previously described conditions.

Metabolite **III** was analyzed by electron impact GC-MS using the same chromatographic conditions as used for metabolite **IV**. No derivative of this metabolite was prepared.

Analysis of Tissues. *Phenyl Goat.* Liver, muscle, kidney, and fat were lyophilized prior to homogenization in a Waring Blendor. An aliquot of each tissue (up to 10 g) was sequentially extracted with hexane, methylene chloride, and methanol. Each liver extract was individually reduced in volume by rotary evaporation at 40 °C. The liver hexane extract was partitioned three times with acetonitrile to remove oils. These acetonitrile and hexane partitions, the methylene chloride extract, and the methanol extract were separately applied as a band to a silica gel thin layer and developed in hexane. Radioactivity remaining at the origin was removed and analyzed in HPLC systems 1 and 2 (all fractions) and HPLC system 4 (acetonitrile, hexane, and methylene chloride fractions only).

Hexane, methylene chloride, and methanol extracts from the kidney and muscle were analyzed by TLC system 1 without purification. The hexane and methylene chloride fat extracts were extracted with acetonitrile as was the liver hexane extract. HPLC system 4 was used for analysis of all of the fat extracts. For chromatographic comparison authentic standards of metabolites **II**, **IV**, and **VI** were also analyzed.

Triazole Goat. Liver, kidney, fat, and muscle were extracted in the same manner as the phenyl goat tissues. Extracts were analyzed in TLC systems 1 and 2 along with authentic standards. After development, radioactive bands were visualized by autoradiography and quantified by LSC.

Table 2. Levels of Radioactive Residues in the Milk of Goats Dosed with either [Phenyl(U)-^{14}C]flusilazole or [Triazole(3)-^{14}C]flusilazole

	phenyl goat		triazole goat	
day	volume (mL)	concentration (mg/kg[a])	volume (mL)	concentration (mg/kg[a])
1	845	0.11	1100	0.36
2	920	0.09	1010	0.66
3	1160	0.09	980	0.74
4	1100	0.27	1020	0.74
5	360	0.74	1010	0.63
6	165	0.74		

[a] Milligrams of flusilazole equivalents per kilogram.

Milk Analysis. *Phenyl Goat.* Milk (100 mL) was mixed with an equal volume of methanol to precipitate proteins (which were removed by centrifugation; approximately 94% of the original radioactivity in the milk remained in solution). The pellet was washed with methanol and the methanol wash added to the supernatant before concentration to dryness by rotary evaporation (45 °C). On average, 85% of the radioactivity in the supernatant was present in the soluble fraction.

The dried residue was extracted repeatedly with methanol. These methanol extracts were combined, concentrated to approximately 10 mL by rotary evaporation (45 °C), and further concentrated under a stream of nitrogen before they were analyzed in TLC system 1.

Triazole Goat. A 5 mL aliquot of each daily milk sample was diluted with an equal volume of methanol and the resultant precipitate was removed by centrifugation; approximately 97% of the radioactivity originally in the milk remained in the supernatant. The supernatant was reduced to dryness through rotary evaporation and the dried residue redissolved in a few milliliters of 0.1 M HCl. Radioactivity was retained on 0.1 M HCl equilibrated Dowex 50 W−X8 resin and then washed sequentially with 0.1 N HCl, distilled water, and 2 M $(NH_4)_2CO_3$.

The ammonium carbonate wash (containing approximately 80% of the radioactivity) was reduced to dryness and radioactivity removed from the solids with methanol. After concentration of the methanol washes to a volume less than 1 mL, radioactive components in an aliquot were separated using TLC system 2. After development, radioactivity on the plate was located by autoradiography on Kodak AR-5 X-ray film. Silica gel associated with radioactive bands visualized in this way were scraped from the plate, and the radioactivity was quantified by LSC in a water/Formula 947 scintillation cocktail (DuPont NEN Products, Boston, MA) gel.

RESULTS

Phenyl Goat. *Distribution of [^{14}C] Residues.* Of the radioactivity administered as [phenyl(U)-^{14}C]flusilazole to the goat, 44.7% was excreted in the urine and 8.1% in the feces. Milk collected during the dosing phase contained only 0.34% of the total administered radioactivity. Tissues collected after sacrifice contained 8.2% of the dose. No attempt was made to calculate the total amount of radioactivity associated with all tissues from the goat.

Concentration of [^{14}C] Residues in Milk and Tissues. [^{14}C] Residues in milk ranged from 0.09 to 0.74 mg/kg (Table 2). Increases in the concentration of [^{14}C] residues in the milk were usually accompanied by lower milk production such that the total amount of radioactive material secreted in the milk each day did not vary as much as did the concentration.

The highest concentrations of radiolabel in edible tissues were in liver and kidney containing, respectively, 13.5 and 8.74 mg/kg (Table 3). Levels of radioactivity in the various muscles were similar. Loin, flank, and

Table 3. Levels of Radioactive Residues in the Tissues of Goats Dosed with either [Phenyl(U)-^{14}C]flusilazole or [Triazole(3)-^{14}C]flusilazole

	concentration (mg/kg[a])	
tissue	phenyl goat	triazole goat
liver	13.5	3.54
kidney	8.74	0.75
muscle[b]	0.52 ± 0.16	0.53 ± 0.01
fat[c]	4.45 ± 0.50	0.40 ± 0.44

[a] Milligrams of flusilazole equivalents per kilogram. [b] Average of concentration in leg, flank, and loin muscle. [c] Average concentration in back, omental, renal, and peripheral fat.

leg muscle contained on an average 0.52 mg/kg total radiolabeled residues. Fat samples contained on an average 4.55 mg/kg total residues.

Identification of Major Residues in the Urine. Examination of the urine using TLC system 1 indicated that extensive metabolism of flusilazole had occurred. Little radioactivity was associated with flusilazole on the TLC plate, even though urine contained a large fraction of the total dosed radioactivity.

Radioactivity in the urine was associated with primarily two compounds (metabolites **II** and **V**), separable by TLC system 1. Metabolite **II** had an R_f of approximately 0.75 in TLC system 1, while metabolite **V** was immobile in this same TLC system.

Purified metabolite **II** had a mass spectrum that agreed very well with that of an authentic sample of bis(4-fluorophenyl)(methyl) silanol (Figure 1). Similarly, the TMS derivatives of both compounds produced the same spectra and confirmed the presence of a derivatizable hydroxyl group (data not shown). Both metabolite **II** and the synthetic standard had identical chromatographic properties in TLC system 1 and HPLC systems 1, 2, and 4.

Metabolite **V** did not migrate in TLC system 1. However, upon treatment with β-glucuronidase, it was converted almost entirely to a radiolabeled species (metabolite **IV**) that had an R_f of about 0.63 in TLC system 1 (Figure 2). Purified metabolite **IV** had the same mass spectrum (Figure 3) and chromatographic behavior as those of bis(4-fluorophenyl)(methyl)(silyl)-methanol). The mass spectrum of the TMS derivative confirmed the presence of a single derivatizable hydroxyl group (data not shown).

Urine also contained traces of the condensation product of two molecules of metabolite **II** (oxy[bis[bis-(4-fluorophenyl)(methyl)(silane)]]) (**VI**). The relative amounts of these two compounds appeared to be a function of the sample workup and analysis procedure. Traces of the condensation product would also appear with time in solutions of metabolite **II** used as a standard (both at 1 and 10 mg/mL). Because of the apparent relationship existing between these compounds, levels of metabolites **II** and **VI** were summed when describing levels of these residues in tissues and milk.

Characterization of Residues in Tissues. The majority (89%) of the total radioactive residue (TRR) in the liver was extractable and was composed primarily of metabolite **II** (and metabolite **VI**; Table 4). Intact flusilazole and unretained, relatively polar compounds (eluting between 1 and 3 min in HPLC system 1) comprised most of the remaining extractable radioactivity. Radioactivity listed as "unknown" did not migrate with any standard, but rather was spread diffusely throughout the chromatogram.

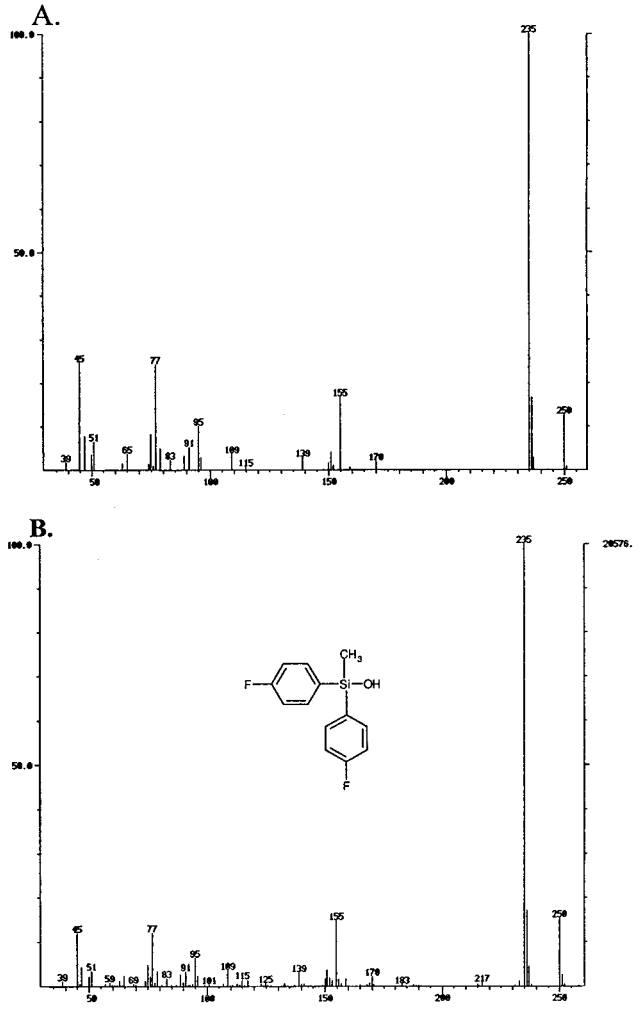

Figure 1. Mass spectral analysis of (A) metabolite **II** isolated from urine and (B) an authentic sample of bis (4-fluorophenyl)-(methyl)silanol.

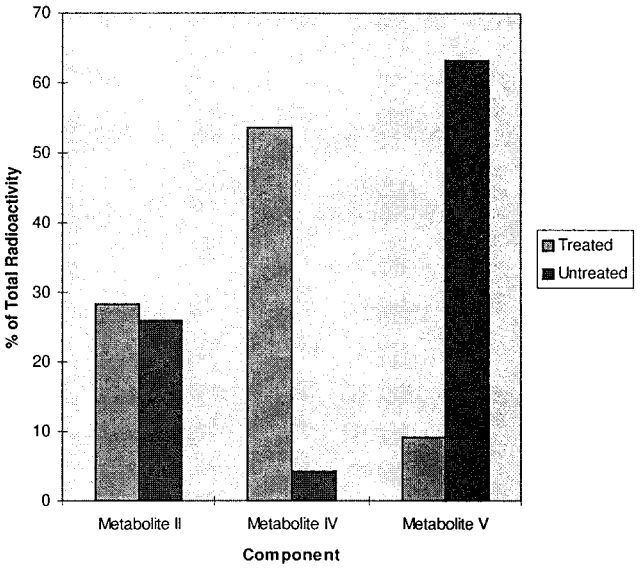

Figure 2. Effect of β-glucuronidase treatment on day 3 urine from phenyl goat.

Multisolvent extraction of the kidney, leg muscle, and back fat was effective at removing the majority of the radioactivity (>96% of the TRR in all tissues). Metabolites **II** (and **VI**) were the major residues in the kidney and fat, while polar residues (unretained in HPLC

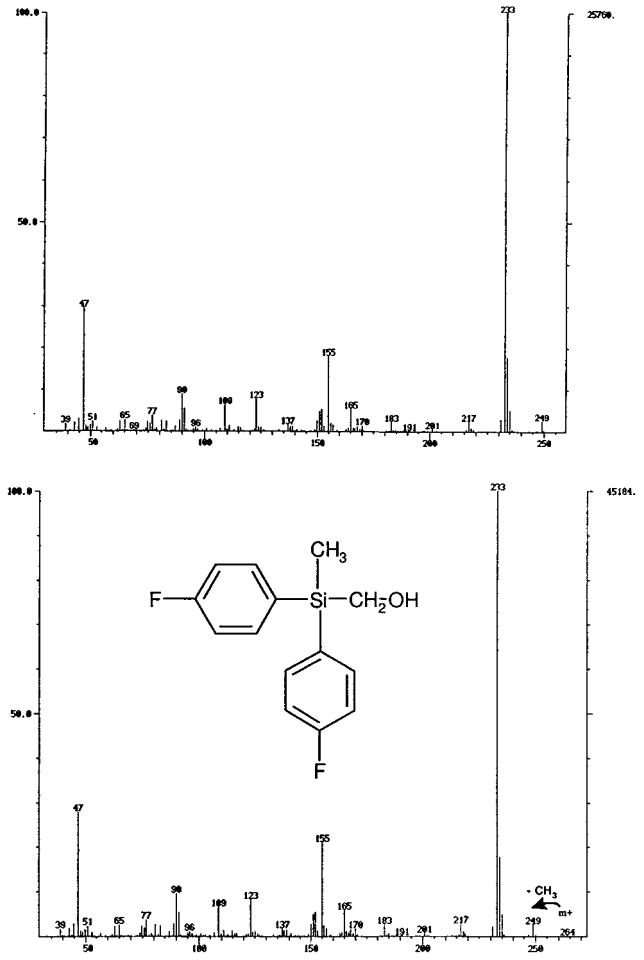

Figure 3. Mass spectral analysis of (A) metabolite **IV** isolated from urine after β-glucuronidase treatment and (B) an authentic sample of bis(4-fluorophenyl)(methyl)(silyl)methanol.

Table 4. Composition of Residues in Selected Tissues and Milk from the Phenyl Goat as a Percentage of Total Radioactive Residue in the Matrix

tissue	II and VI	flusilazole	unidentified	polar[a]	unextracted
liver[b]	58	12	11	8	11
kidney[c]	74	<1	3	20	2
leg muscle[c]	23	5	1	69	3
back fat[d]	73	9	11	5	<1
day 4 milk[e]	58	13	<1	28	

[a] Material which did not migrate in TLC system 1 or was not retained upon HPLC analysis. [b] Analyzed in HPLC system 1 and 2. [c] Analyzed in HPLC system 1. [d] Analyzed in HPLC system 4. [e] Analyzed in TLC system 1.

system 1 or immobile in TLC system 1) comprised the major residues in leg muscle. Neither metabolite **IV** nor **V** was found in any tissue. Polar residues from kidney, isolated by chromatography in HPLC system 1, were further subjected to chromatography on a 25 cm $\times$ 4.6 mm Zorbax C8 column using a nonlinear gradient system starting at low solvent strength (10%) acetonitrile. Six separate fractions were isolated, but insufficient material was present in any fraction to permit identification.

Characterization of Residues in Milk. Results from analysis of day 4 milk are representative of that observed in the other milk samples and are reported in Table 4. Metabolite **II** comprised the majority (about 60%) of the radiolabeled residue in milk, with flusilazole and polar residues making up the remainder. When polar residues were treated with β-glucuronidase, or heated in 0.1 M HCl at 100 °C for 1 h, they did not change their polar nature as assayed in HPLC system 1. Such stability to hydrolysis suggests that the polar residues were not conjugates such as metabolite **V**.

Triazole Goat. *Distribution of [14C] Residues.* When the goat was fed [14C-triazole]flusilazole in five daily doses, 36% of the total administered radioactivity had been excreted at sacrifice. Approximately 23.3% of the total dose appeared in the urine, and 12.8% in the feces. Milk collected over the 5 day dosing period contained 1.3% of the total dose while all the tissues analyzed accounted for 2.5% of the dose.

Concentration of [14C] Residues in Milk and Tissues. Concentrations of the radiolabeled residues in the daily milk samples did not vary to any great extent, nor did the volume of milk output (Table 2). The concentration of radiolabeled residues in the milk reached a plateau of about 0.7 mg/kg. Liver contained the highest concentration of radiolabeled residues (3.54 mg/kg, see Table 3), while kidney had 0.75 mg/kg. All of the muscle tissues analyzed contained roughly the same concentration of radioactivity (average of 0.53 mg/kg in leg, loin, and flank muscle). The average residue in fat was 0.40 mg/kg and ranged from a high of 0.94 mg/kg in back fat to 0.26 mg/kg in both the renal and omental fat.

Characterization of Major Residues in the Urine. Urine contained one major radiolabeled component when analyzed in TLC system 2 (Figure 4). Capillary GC-MS analysis of the purified compound (metabolite **III**) showed it to have the same mass spectrum as authentic 1H-1,2,4-triazole (Figure 5). Both isolated metabolite **III**, and the synthetic standard of 1H-1,2,4-triazole comigrated by TLC (data not shown).

Characterization of Major Metabolites in Milk. Metabolite **III** comprised the bulk of the radioactivity in milk, along with low levels of flusilazole. In milk, metabolite **III** accounted for 87−99% of the radioactivity (day 4 analyses only in Table 5).

Characterization of Major Residues in the Tissues. The sequential three solvent extraction procedure (hexane, methylene chloride, and methanol) removed 90% or more of the radioactivity from the goat tissues.

The composition of the radioactivity in these tissues is summarized in Table 5. In the liver, 76% of the TRR (2.7 mg/kg) was recovered as intact flusilazole (this compares with 1.6 mg/kg flusilazole in the liver from the phenyl goat). Metabolite **III** was the predominant metabolite in the muscle, fat, and kidney.

DISCUSSION

Goats received 5 (or 6) oral doses of [14C]flusilazole in an attempt to achieve a plateau in the level of total radioactive residues in the milk. Results in Table 2 indicate that this plateau was reached almost immediately in the phenyl goat, and within 2 days in the triazole goat. The increased concentrations on the last days of phenyl goat dosing were accompanied by a sharp decrease in milk production. The reason for the decreased milk production is unknown. Both the phenyl and triazole goats were sacrificed within 24 h of the last dose. While two different sacrifice times were selected (respectively, 10 h after the final dose and 22 h after the final dose for the phenyl and triazole goats), both sacrifice times resulted in sufficient tissue residues to permit identification of the terminal residues in the

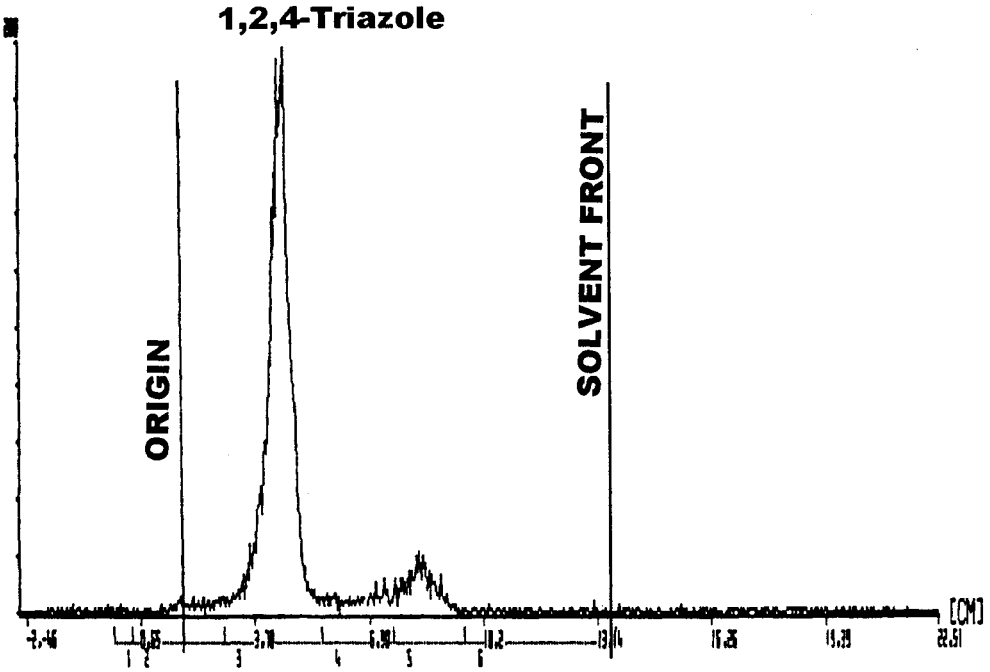

Figure 4. Analysis of triazole goat urine in TLC system 2.

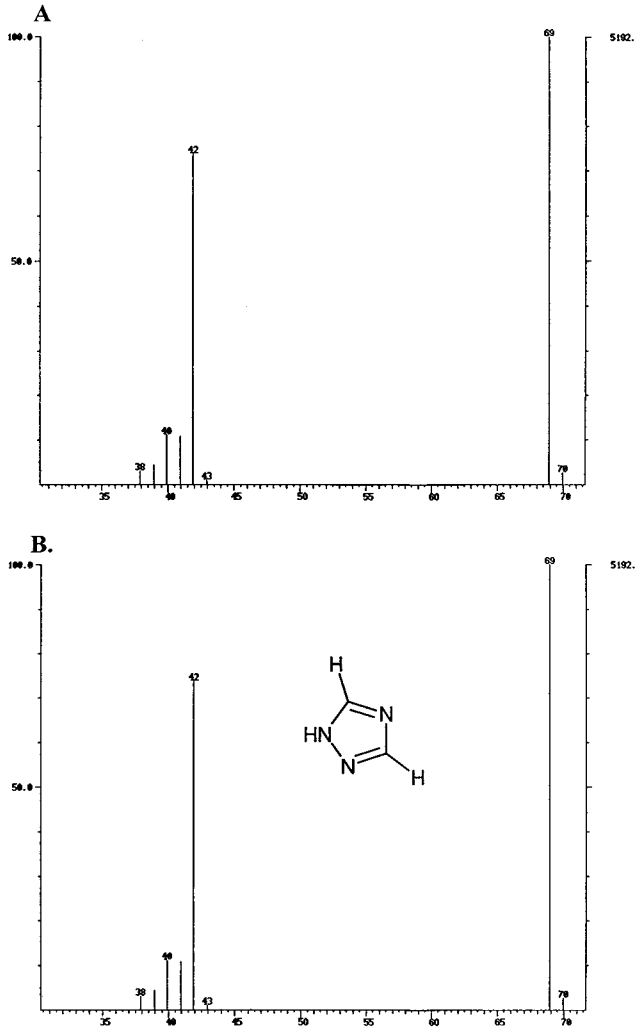

Figure 5. Mass spectral analysis of (A) metabolite **III** isolated from urine and (B) an authentic sample of 1*H*-1,2,4-triazole.

edible goat tissues. Most of the recovered radioactivity was found in the excreta of either goat. Of that retained in tissues or milk, concentrations in the tissues and milk of the triazole goat (calculated as flusilazole equivalents) were generally less than residue levels in the corresponding tissues from the phenyl goat. The remainder of the radioactivity administered to the dose was unaccounted for and is believed to be still retained in the digestive tract (and therefore not quantified by combustion analysis) or lost as a volatile metabolite. Data already presented have shown that flusilazole was extensively metabolized to metabolites **II**, **IV**, and **V** which have lost the triazole functional group. Derivatized triazoles have been found in other studies to be extensively metabolized to CO_2. For example, 1-amino-1,2,4-triazole (Amitrole) is extensively metabolized to CO_2 by microorganisms (Menzie, 1969). The rich microbial population in the rumen of the goat may be expected to accomplish a similar transformation of 1H-1,2,4-trizole released on formation of metabolites **II** and **IV**. Evidence that the triazole portion of flusilazole can be metabolized to CO_2 was found in a study investigating the fate of flusilazole in rats (Anderson et al., 1986). No CO_2 was recovered from rats dosed with [phenyl-(U)-^{14}C]flusilazole, but CO_2 was recovered from rats dosed with [^{14}C-3-triazole]flusilazole.

Table 5. Composition of Residues in Selected Tissues and Milk from the Triazole Goat (as a Percentage of the Total Radioactive Residue in the Matrix)

tissue	flusilazole	III	polar[a]	unidentified[b]	unextracted	na[c]
liver	76	14	3	1	6	<1
kidney	<1	72	11	<1	7	11
leg muscle	<1	62	11	15	10	2
back fat	<1	53	15	<1	7	24
day 4 milk	<1	>99				

[a] Material remaining at the origin of TLC system 2. [b] Includes a radiolabeled species in the liver hexane extract that had an R_f of 0.57 in TLC system 2 and radiolabeled species present in the leg muscle methanol extract that had R_f's of 0.07 and 0.25 in TLC system 2. [c] Radiolabeled compounds in the hexane and methylene chloride extracts which could not be analyzed because of large amounts of lipids or because the levels of radioactivity were too low.

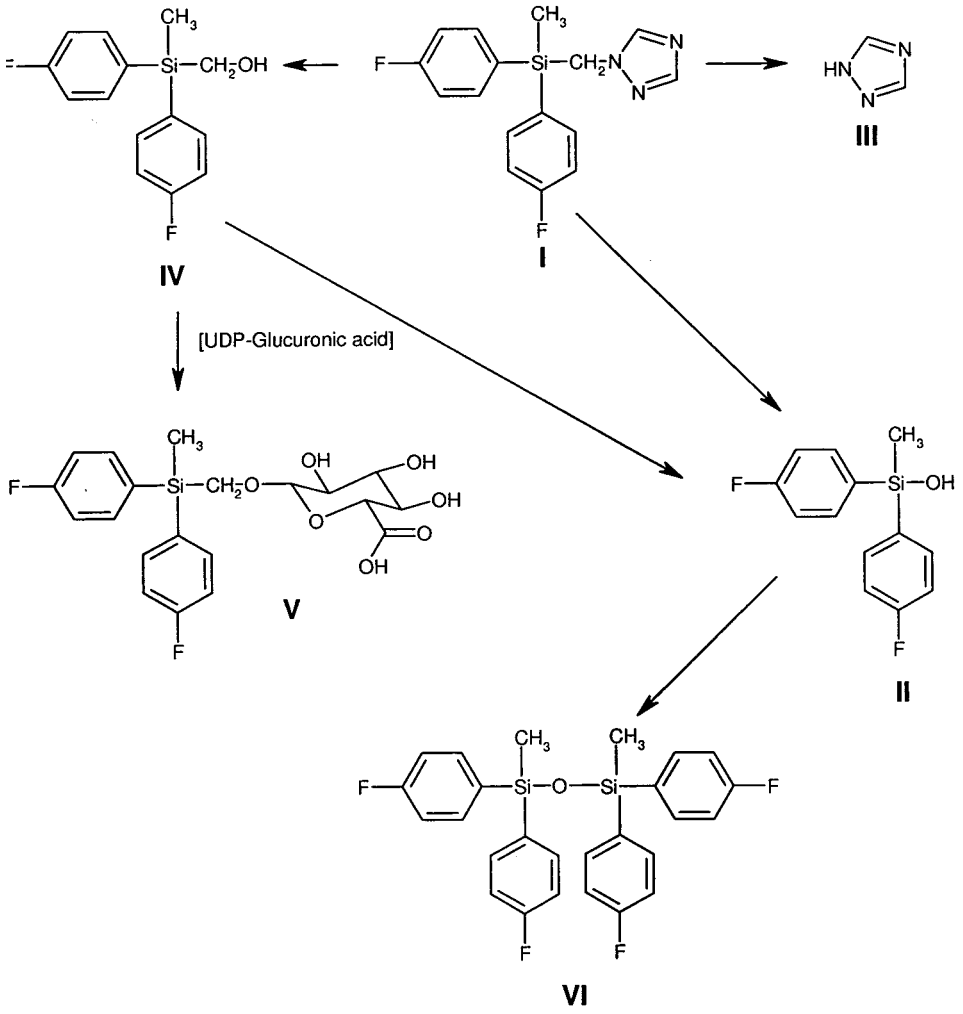

Figure 6. Proposed metabolic pathway of flusilazole in the lactating goat.

Metabolites observed clearly indicate that the "triazole" portion of the molecule was cleaved from the "phenyl" portion in the course of metabolism (a proposed metabolic pathway is presented in Figure 6). Cleavage of the triazole portion of the molecule has been observed in the metabolism of flusilazole in other animal species. In rats (Kaneko et al., 1995) and in hens (Lin, 1988a,b), cleavage of the carbon−nitrogen bond to yield 1H-1,2,4-triazole and other metabolites containing the phenyl portion of the molecule was the major degradative pathway.

Cleavage of triazole fungicides, resulting in the release of 1,2,4-triazole, is not consistently seen in the metabolism of this class of fungicides by mammals. For example, metabolism of propiconazole (1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolane-2-yl]methyl]-1H-1,2,4-trizole in rats and mice (Bissig and Muecke, 1988) and in swine, cows, and poultry (Kaneko et al., 1995) saw no evidence of cleavage of the triazole moiety, although there was limited oxidation of the triazole. Similarly, no loss of triazole was seen in the metabolism of the triazole fungicides bitertanol, diniconazole, triadimefon, triadimenol, and paclobutrazole in rats. Nor did metabolism of fluconazole in humans (reportedly similar to that in laboratory animals; Brammer et al., 1991) involve cleavage of the triazole moiety, but rather N-oxidation on the triazole ring.

However, metabolism of hexaconazole (Kaneko et al., 1995) and DTP (dichlorophenyl-bis-triazolylpropanol, a compound structurally related to ketaconazole; Bomont et al., 1994) in rats did result in the loss of the triazole group. DTP was either excreted in urine unchanged or underwent an oxidation reaction in which one molecule of parent was N-dealkylated to yield one triazole and one diol entity. The diol can be glucuronidated before excretion or oxidized to the acid. Possibly the ability to oxidatively remove the triazole functionality is related to the presence of a methylene group immediately adjacent to the heterocycle, or more likely a result of a preferred confirmation at the oxidative enzyme's active site.

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