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Analysis of Tissue Residues and Comparative Metabolism of Virginiamycin in Rats, Turkeys, and Cattle

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Liver tissue samples from turkeys, cattle, and rats given [^{14}C]virginiamycin were examined for the presence of metabolites. Extraction of the liver was performed sequentially with methanol and pH 7.4 phosphate buffer. The methanol fraction was further partitioned into chloroform-soluble and water-soluble fractions. The majority of the total liver residue (56–73%) remained intractable following these treatments. The three extracts were fractionated by normal- or reversed-phase HPLC. The results indicated that virginiamycin was metabolized to a large number of fragments and that no single metabolite represented greater than 3.5% of the total liver residue. Due to sample limitations no metabolite identification was possible at this time. Fortification experiments indicated that little, if any, parent virginiamycin was present in the tissue. Additional balance–excretion studies conducted in cattle and rats demonstrated that the majority of the dose (83–94%) was eliminated in the feces.

Virginiamycin is produced by a mutant of *Streptomyces virginiae* and is active against gram-positive bacteria of the gut (DeSomer and Van Dijck, 1955). The antibiotic is composed of two major factors (M_1 and S_1) functioning synergistically when combined in the optimum ratio of 4:1 (M to S). The structures of factors M_1 and S_1 are shown in Figure 1.

Favorable responses in growth and/or feed efficiency have been reported when virginiamycin is supplemented in the diets of broilers (March et al., 1978; Miles, 1982; Miles et al., 1984), turkey poults (Buresh et al., 1984), and swine (Maxwell, 1984). The compound has also been shown to be effective against necrotic enteritis in broilers (Daniels, 1984) and in the management of swine dysentery (Miller et al., 1972).

We have undertaken several studies comparing the virginiamycin metabolic profile in liver tissue from rats, turkeys, and cattle. In conjunction with these studies, additional data concerning the excretion profile in rats and cattle were also obtained. The results of these investigations indicate that the metabolic profile for virginiamycin is similar among the three species studied and that the antibiotic appears to be extensively metabolized, with little, if any, parent compound being present in the tissues.

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REAGENTS

Test Substance. The production of [^{14}C]virginiamycin is via fermentation with *S. virginiae* using radiolabeled sodium acetate, glycine, proline, lysine, and phenylalanine as precursors. Both factors become relatively uniformly labeled by this procedure. The fermentation broth is extracted with hexane to remove oils followed by ethyl acetate which contains the crude product. The solvent is evaporated, redissolved in chloroform, and purified by preparative HPLC (Lichrosorb SI-60, 10 μm, 23 mm × 50 cm column, ES Industries; chloroform–methanol–trifluoroacetic acid (98.5:1.5:0.02) mobile phase; 20 mL/min flow rate; 330-nm UV detection). Fractions cooled in dry ice were collected (factor S, 8–10 min; factor M 14–17 min) and flash evaporated. Final purification of the individual factors was achieved by recrystallization from hot methanol

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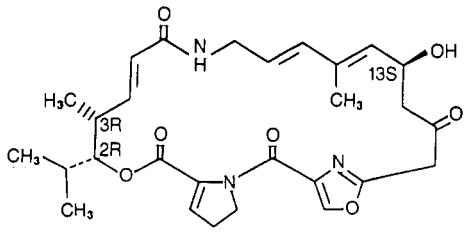
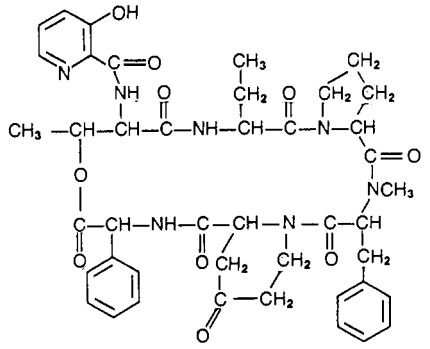


Factor M_1



Factor S_1

Figure 1. Chemical structure of virginiamycin factors M and S.

(factor S) and precipitation with petroleum ether from a concentrated chloroform solution (factor M). The separated factors were recombined in a 4:1 (w/w) ratio. Appropriate amounts of nonradiolabeled reference standards were added to adjust each factor to identical specific activities. The final [^{14}C]virginiamycin assayed to a radiochemical and chemical purity of ≥98% (HPLC, as above except with 5 μm, 4.6 mm × 25 cm column, 1 mL/min flow rate, 297-nm UV detection; TLC, chloroform–methanol (95:5) or ethyl acetate–methanol–ammonium hydroxide (100:10:5)). The microbiological potency assayed to 193% using a pad plate diffusion assay with *Corynebacterium xerosis*. Note: The *original* analytical standard for virginiamycin was based on activity only, and a potency was set at 100% without rigorous chemical purity characterization. Chemically pure virginiamycin (as described here) is approximately twice as active (for an equivalent sample weight) as the original standard. New preparations of VM are still assayed vs. the original activity standard and thus will always assay with activities of 190–200%.

PROCEDURE

Balance–Excretion Study. *Rats.* Six (3 M, 3 F) Sprague–Dawley rats, 180–200 g, were purchased from Charles River Laboratories, Inc. Upon arrival the rats were placed in individual metabolism cages designed for the separate collection or urine and feces. Food and water were given ad libitum, and acclimation to the cages was continued for 1 week. Following acclimation, the rats were given a single dose of [^{14}C]virginiamycin (0.803 mCi/g) suspended in soybean oil (15 mg/mL; 25 mg/kg of body weight). Urine and feces were collected separately from each rat daily for 4 days. Aliquots of urine (1.0 mL) were counted directly in 10-mL Instagel (Packard), and fecal samples (0.5 g) were assayed by combustion (Packard Model 306C sample oxidizer). After the fourth collection period, the rats were sacrificed by cervical dislocation, and their liver and kidneys were removed, rinsed with H_2O, blotted dry, immediately frozen, and stored until assayed. The tissues were cut into 0.5–1.0-g samples, and the total radioactivity content was determined by combustion.

Cattle. Three (2 M, 1 F) Friesan–Hereford strain beef cattle (300 kg, 18 months) were acclimated on-site (Huntingdon Research Center, Cambridgeshire, England) for a period of 2 weeks. The animals were subsequently transferred to individual pens and given oral doses of nonradioactive virginiamycin at a dose level of 1 mg/kg of body weight per day for 14 days. The daily dose was administered in two equal parts at 12-h intervals in gelatin capsules. On day 15 the animals were given a single oral dose of [^{14}C]virginiamycin (0.193 mCi/g) at the same dose level. Urine was collected for 7 days from each animal by means of a bladder catheter. Feces were collected at 12-h intervals with a harness and collecting bag. Urine was assayed directly by liquid scintillation counting (LSC) while feces were assayed by LSC (methanol extract) and combustion (residue).

Metabolism Studies. *Turkeys.* Thirty-day-old turkey chicks were obtained from Godshall's Hatchery, Inc., Souderton, PA, placed two to four per cage for the first 7 days, and given food and water ad libitum. Subsequently the birds were housed individually, and daily feed intakes were recorded and continued for the duration of the study. Lighting was maintained continually. The room temperature was 88–90 °F for the initial 10 days and subsequently reduced 5 °F per week until a maintenance level of approximately 65 °F was attained. After the acclimation period (21 days postarrival), 16 birds from the midweight range were selected and randomly assigned to two groups. Group I (4 M, 4 F) received [^{14}C]virginiamycin-medicated feed while group II (4 M, 4 F) served as the controls. The birds were maintained on a commercial turkey-starter ration (Pennfield Feeds, Corp.) until sacrifice. Group I received feed medicated with [^{14}C]virginiamycin (0.283 mCi/g) at a level of 50 g/ton, microbiological activity basis (approximately equal to 25 g/ton, weight basis). The control group was maintained on the unmedicated basal ration.

All birds were sacrificed by cervical dislocation after 43 days (postacclimation). Medicated feed was removed 6 h prior to sacrifice to simulate travel time to the abattoir. After sacrifice, the entire liver was removed, immediately frozen, and stored until assayed. Tissue samples (0.5–1.0 g) were assayed for total radioactivty content by combustion (Packard Tri-Carb Sample Oxidizer, Model 306) followed by LSC (Packard Tri-Carb, Model 460C).

Rats. Six rats (3 M, 3 F; 165–185 g) were purchased and acclimated to metabolism cages as described in the balance–excretion study. The rats were dosed with [^{14}C]-virginiamycin (0.803 mCi/g) suspended in soybean oil (25 mg/kg; 15 mg/mL) once daily for 14 consecutive days. An additional group of rats (3 M, 3 F) were dosed with vehicle only and served as controls. All rats were sacrificed 6 h following the final dose, and the livers were removed for combustion assay and extraction.

Cattle. Three (2 M, 1 F) Friesan–Hereford strain beef cattle were purchased, acclimated, and dosed with nonradioactive virginiamycin as described for the balance–excretion study. The animals were subsequently dosed with [^{14}C]virginiamycin (0.193 mCi/g) daily (two equal parts at 12-h intervals) for 7 days. The cattle were sacrificed (Rompun (pentobarbitone) exsanguination) 10 h after the final radioactive dose. The livers were removed, frozen on dry ice, and shipped to SmithKline Animal Health Products, Applebrook Research Center, West Chester, PA, for combustion assay and extraction.

Tissue Extraction. The male and female dosed turkey livers (metabolism study) were pooled by sex and homo-

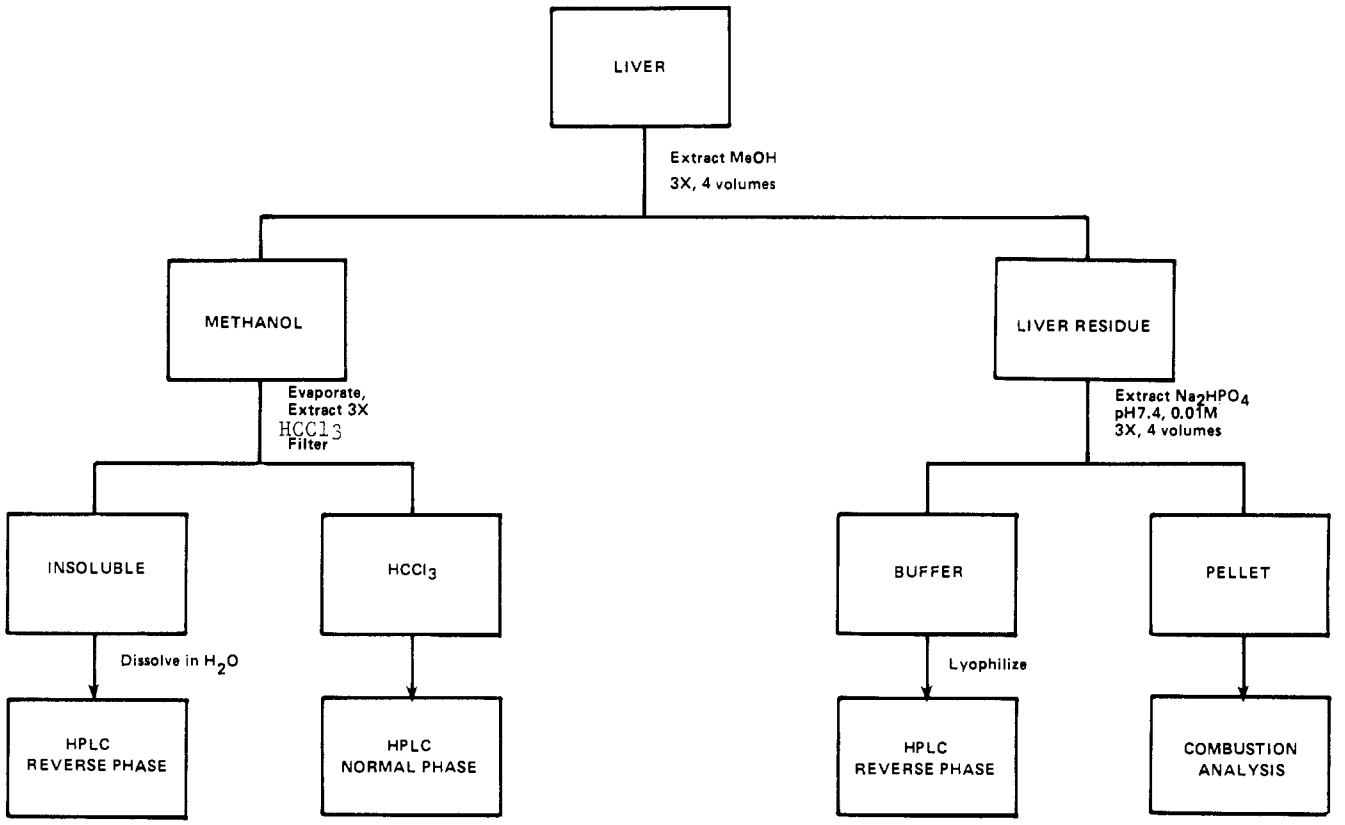


Figure 2. Extraction procedure flow chart.

genized in a blender. Approximately 65 g of pooled homogenate from each sample was further homogenized (Brinkman, Polytron) with 4 volumes of methanol. After centrifugation (40000*g*, 10 min) the supernatant was decanted and the extraction repeated two times. The combined methanol extracts were assayed (LSC), evaporated to near dryness on a rotary evaporator, and lyophilized overnight. The methanol-extracted liver tissue was further homogenized (3×, 4 volumes) with 0.01 M Na_2PO_4, pH 7.4. Aliquots of the buffer extract were removed for counting, and the remainder was lyophilized to dryness. The residual liver "pellet" was also lyophilized and assayed for remaining radioactivity by combustion. Dry chloroform (50 mL) was added to the lyophilized methanol extract and the soluble material separated by filtration through Whatman No. 4 filter paper (Buchner funnel). Additional chloroform was used for washing until the filtrate was clear. The insoluble material remaining on the filter paper was dried by continued aspiration, dissolved in distilled water, and filtered. Additional water was used as necessary to totally dissolve the residue. Aliquots (3–10 mL) of both extracts were removed for counting. The water fraction was lyophilized to dryness. The chloroform extract was concentrated to approximately 50 mL on a rotary evaporator, filtered through a 0.45- μm filter, and stored at 4 °C until analyzed by HPLC. Prior to HPLC analysis of the water fraction and phosphate buffer extracts, the lyophilized samples were redissolved in distilled water (50 mL) and filtered. (Note: The procedure described above is for turkey liver; however, both rat and cattle liver tissue samples were processed through a similar procedure. Comparative extraction and chromotography data are indicated in the results where appropriate). The overall extraction scheme is illustrated in Figure 2.

In a separate experiment, [^{14}C]virginiamycin (0.283 mCi/g; 31 641 dpm) was added to a 20-g sample of control turkey liver (male). The fortified sample was processed through the tissue extraction procedure described above. In addition, the chloroform extract from this tissue sample was processed through the identical HPLC procedure (described below) as the corresponding extracts from livers of the birds that consumed the medicated feed.

HPLC. The equipment used consisted of two Waters Model 590 solvent delivery module pumps, equipped with extended flow heads; a Waters Model 721 system controller; a Waters Model 481 variable-wavelength UV detector equipped with a preparative flow cell; a Hewlett-Packard Model 3392A integrator; a Rheodyne injector valve, Model 5701, equipped with solenoids for pneumatic activated injections, Model 7163; an Autochrom solvent select valve, and a 55-mL sample loop.

The $HCCl_3$ extract was analyzed on a 10-μm Chromegasorb SI-60 column, 50 cm × 23 mm (ES Industries, Marlton, NJ) with a flow rate of 22.5 mL/min. A saturator precolumn and a guard column (7 cm × 1 cm) consisting of the same packing material were also employed. The solvent program was as follows: linear gradient from 100% $HCCl_3$ to 50:50 (v/v) MeOH–$HCCl_3$ over 15 min; linear gradient to 80:20 (v/v) MeOH–$HCCl_3$ over 5 min; maintainence at 80% MeOH–$HCCl_3$ for 20 min. Nine fractions were collected as indicated on the chromatogram (Figure 3a). Ultraviolet absorbance was monitored at 254 nm. The sample injection volumes were 10–15 mL and were repeated until the entire extract had been processed. The collected fractions were evaporated to dryness and assayed for radioactivity by LSC.

The water fraction and buffer extract were analyzed on a 10-μm Chromegabond MC-18 column, 30 cm × 23 mm (ES Industries, Marlton, NJ) with a flow rate of 22.5 mL/min. A guard column containing the same packing material (7 cm × 1 cm) was also employed. The solvent program was as follows: 100% $(NH_4)_2CO_3$ buffer (0.01 M, pH 6.8–6.9) for 5 min; initiate gradient to 15% isopropyl alcohol–buffer over 12.5 min; maintainence at 15% iso-

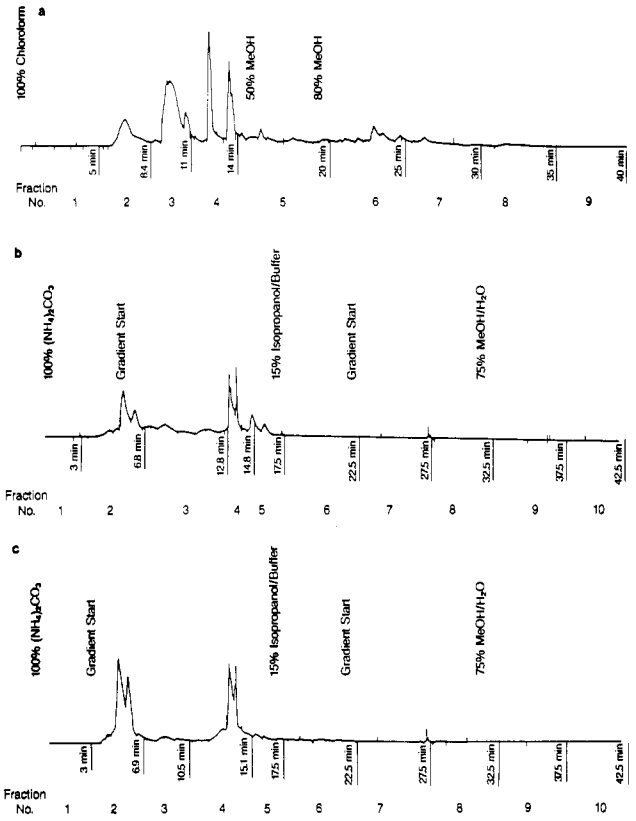


Figure 3. Chromatographic profiles from turkey liver: (a) $CHCl_3$ extract; (b) H_2O extract; (c) buffer extract.

Table I. Excretion Profile and Tissue Residues from Rats and Cattle Dosed with [^{14}C]Virginiamycin at 25 and 1 mg/kg, Respectively

	% initial dose			
	rats		cattle	
sample	males	females	males	females
feces	87.5	89.2	83.8	94.2
urine	2.1	2.4	1.7	1.3
liver	0.12	0.08	nd	nd
kidney	0.015	0.014	nd	nd

[a] Residue levels correspond to 757 and 525 ppb virginiamycin equivalents, respectively, for males and females. [b] Residue levels correspond to 283 and 224 ppb virginiamycin equivalents, respectively, for males and females. [c] Data from one animal only.

propyl alcohol–buffer for 5 min; initiate linear gradient to 75% MeOH–H_2O for 10 min; maintainence at 75% MeOH–H_2O for 10 min. The $(NH_4)_2CO_3$ buffer was prepared by dissolving the appropriate amount of salt in distilled water, adding dry ice to reduce the pH to 5.5–6.0, filtering, and degassing the solution until the final desired pH was obtained. Ten fractions were collected as indicated on the chromatograms (Figure 3b,c). Ultraviolet absorbance was monitored at 280 nm. The sample injection volumes were 10–15 mL and were repeated until the entire extract had been processed. The collected fractions were taken to dryness by evaporation (rotavap) and/or lyophilization and redissolved in a minimum amount of distilled water. Aliquots were assayed for radioactivity content by LSC.

RESULTS AND DISCUSSION

The results of the balance–excretion studies in rats and cattle are shown in Table I. The bulk of the radioactivity was found in the feces while only a small percentage of the dose (<3%) was excreted via the urine. The majority of the administered dose was eliminated during the first 48 h. The total residue remaining in the rat liver tissue after 96 h accounted for only a small percentage of the administered dose as indicated in Table I. The liver residues found in the male rats were significantly higher ($p < 0.05$) than those found in the females while no sex difference in the kidney residue levels was observed.

Table II. Percentage of [^{14}C]Virginiamycin Residues Found in the Various Organic and Aqueous Fractions following Extraction from Liver Tissue of Turkeys, Rats, and Cattle[a]

	% recovered radioactivity		
extract	rat	turkey	cattle
methanol	18.3	24.5	35.6
$HCCl_3$ sol[b]	4.6 (25)	9.8 (40)	12.1 (34)
H_2O sol[b]	13.7 (75)	14.7 (60)	23.5 (66)
buffer	9.1	12.4	8.4
pellet	72.6	63.0	56.0

[a] Extraction procedure according to Figure 2 and as described in methods. [b] Data in parentheses indicate the percentages of the initial methanol extracts.

The relatively high ppb levels (up to 757 ppb in liver) seen in the rat (Table I) following a 4-day withdrawal period was a reflection of the substantially higher administered dose (25 mg/kg). Although tissue residues for the cattle (Table I) were not determined in this case, data from previous studies are available. For comparison, the turkey livers had total residues of approximately 300 ppb and cattle treated at 1 mg/kg per day for 7 days with [^{14}C]VM (0-day withdrawal) had liver residues of approximately 200–300 ppb. These levels are considerably lower than those observed for the rats.

Liver tissue samples from turkeys, rats, and cattle were processed through the extraction procedure illustrated in Figure 2. Liver was chosen for study since this tissue was found to contain the highest residues in all species (target tissue). The percentage of radioactivity in each of the extracts is shown in Table II. The results indicate that the majority of the tissue radioactivity remained in the pellet after all the extraction procedures were completed. Attempts were made to remove additional radioactivity by employing additional organic solvents (butyl alcohol, ethyl acetate) or by preparing aqueous buffers having alternate pH values (3.0–9.0). None of these techniques resulted in the solubilization of additional radioactivity. Although solubilization was attainable by strong-acid hydrolysis, this procedure was not pursued due to the likelihood of substantially altering the drug residues from their original form. The pellet residues were thus considered intractable by ordinary chemical means.

The extractable residues were partitioned among three fractions: $HCCl_3$, H_2O, buffer (Figure 2). The initial use of methanol resulted in the removal of a larger percentage of radioactivity than if $HCCl_3$ alone or MeOH/$HCCl_3$ combinations were used. Although slight variations were apparent, the data in Table II indicate that the virginiamycin residues partitioned similarly among the liver extracts from these three species.

The low total residue levels in the liver tissue of the three species along with the low percentage of extractable residues precluded any attempt at metabolite identification in the present study. However, everal experiments were performed in an attempt to (1) determine the percentage of the total liver residues that might result from parent virginiamycin and (2) demonstrate that the extractable radioactivity was composed of multiple components.

The fortified control turkey liver homogenate was processed through the developed tissue extraction procedure (see Figure 2 and methods). The percentage of the total recovered radioactivity in each fraction is shown in Table

Table III. Percentage of [^{14}C]Virginiamycin Found in the Various Organic and Aqueous Fractions following Extraction of Fortified Control Turkey Liver[a]

extract	% rec radioact	extract	% rec radioact
methanol	97.2	buffer	0.1
$HCCl_3$ sol	84.2	pellet	2.7
H_2O sol	15.8		

[a] Extraction procedure according to Figure 2 and as described in methods.

III. The results indicate that the majority of the radioactivity (84.2%) was extracted into the $HCCl_3$ fraction while virtually none (2.7%) was found in the pellet. This is in marked contrast to the data in Table II and indicates that little, if any, of the residues present in the rat, cattle, or turkey livers are likely to be parent virginiamycin.

Evidence for the multiple-component nature of the extractable tissue residues was obtained using HPLC. Each of the three extracts ($HCCl_3$, H_2O, buffer) was processed through their respective gradient procedures (see methods). Control tissue extracts were processed as well. In all cases, the absorbance chromatograms of the control and dosed tissue samples appeared similar, indicating that only natural tissue components were being observed. Since the tissue residue levels were extremely low and the injected samples were crude extracts, this was not an unexpected result. The development of the gradient HPLC methods was based on the assumption that if the chromatographic system separated the natural tissue components, separation among the drug-related residues was also likely to be achieved. By processing the dosed tissue extracts and counting the collected fractions, separation of the virginiamycin residues was realized. Sample chromatograms of the three dosed tissue extracts from turkey liver are shown in Figure 3a–c along with the fraction collection intervals. The percentage of the total recovered radioactivity in each fraction is shown in Table IV. When these values are corrected to reflect the percentage of total tissue radioactivity (based on the initial combustion measurements), the results indicate that no single fraction contained greater than 3.5% of the total liver residue. This finding, coupled with the apparent absence of intact virginiamycin, indicates that the antibiotic is extensively metabolized and that the liver residues are comprised of a large number of components. The requirement of such severe gradient conditions for the HPLC (i.e., 0–80% MeOH for the $HCCl_3$ extract) demonstrates that the injected samples were composed of residues that differ greatly with respect to their individual polarity. For reference, both components of standard virginiamycin, if present, would elute in fraction 3 of the chloroform extract (Figure 3a). HPLC of the VM standard in the aqueous system was not performed since only a small percentage of the compound partitions into this fraction following extraction from fortified tissue (Table III). Similar HPLC data (not shown) were generated for the rat and cattle liver extracts during the course of these investigations. The corresponding extracts were fractionated by the identical gradient procedures described for the turkeys, and an analogous separation of radioactive components was obtained. These results emphasize the similarities in the liver residue profile among the three species studied.

Table IV. Percentage of the Total Radioactivity in the Collected HPLC Fractions of the $HCCl_3$, H_2O, and Buffer Extracts from Turkey Liver

	% total recovered radioactivity		
fraction no.	$HCCl_3$ extr[a]	H_2O extr[b]	buffer extr[c]
1	0.0	4.0	0.6
2	0.0	21.3	3.4
3	32.2	0.0	1.2
4	9.0	0.7	15.5
5	11.5	5.9	19.6
6	21.9	25.5	37.9
7	14.4	8.0	7.7
8	7.8	30.7	11.4
9	3.2	3.9	1.3
10		0.0	1.5

[a] Fractions correspond to those shown in Figure 3a. [b] Fractions correspond to those shown in Figure 3b. [c] Fractions correspond to those shown in Figure 3c.

CONCLUSIONS

Virginiamycin is excreted primarily in the feces. The majority of the tissue residues were found to be intractable by solvent and buffer extraction techniques, precluding any attempts at metabolite isolation and identification at this time. The extractable residues were shown, however, to consist of multiple components, each of which accounted for a maximum of 3.5% of the total liver residue. Parent virginiamycin was not a major tissue residue since the extraction profile of a fortified control liver sample was significantly different from that of the dosed tissues. The residue profile for virginiamycin was similar among the three species studied (rats, turkeys, cattle). The tissue analyses presented here indicate that virginiamycin is extensively metabolized and that no single metabolite comprises a substantial portion of the total residue. The low total tissue residues of virginiamycin coupled with its apparent extensive metabolism are highly desirable qualities for a drug used in food-producing animals.

Registry No. Virginiamycin, 11006-76-1.

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