ENVIRONMENTAL ASSESSMENT FOR VIRGINIAMYCIN IN CATTLE

1. DATE: October 22, 1993

2. APPLICANT: SmithKline Beecham Animal Health

3. ADDRESS OF APPLICANT: SmithKline Beecham Animal Health
1600 Paoli Pike
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4. DESCRIPTION OF THE PROPOSED ACTION:

A. Description of the Requested Approval

SmithKline Beecham Animal Health is requesting approval for a New Animal Drug Application to NADA 140-998 for the use of virginiamycin (Vm) in cattle. This animal drug is currently approved, under NADA 91-467 for the following indications: for treatment and control of swine dysentery, increased rate of weight gain and improved feed efficiency in swine, turkeys and broiler chickens and prevention of necrotic enteritis caused by **Clostridium perfringens** in broiler chickens. This application provides for increased rate of weight gain, improved feed efficiency and reduction in the incidence of liver abscesses in cattle at a maximum dose level of 25 g/ton (on a 100% dry matter basis) using previously approved Stafac® 500, 50, 20, and 10 Type A Medicated Articles. Refer to 21 CFR 558.635 for approved species, uses and use levels for virginiamycin. The trade name of the product to be used in cattle is V-Max™ Type A Medicated Article. No changes are proposed for the manufacturing, formulation, production methods or dosage forms currently approved.

B. Need for the Proposed Action

Controlled clinical studies have demonstrated that virginiamycin increases the growth rate and the feed efficiency in cattle. This provides a lower unit production cost and increased availability of beef to the consumer at a lower cost.

C. Production Location

Virginiamycin will continue to be produced in Genval, Belgium, by Recherche et Industrie Therapeutiques (RIT), S.A., a wholly owned subsidiary of SmithKline Beecham Corporation. Manufacturing of premixes (Type A Medicated Articles) will continue at our facility in Omaha, Nebraska.

D. Environments which will be Affected

Since virginiamycin is a growth enhancer proposed for use in cattle feed, the geographic areas of predominant usage will naturally coincide with the areas of greatest commercial cattle feedlot production. The following

table lists the relative number of cattle raised by each state as compared to the rest of the country. 000012

Table 1

U.S. Department of Agriculture, "Cattle on Feed" Report
January 1987

	Farmer Feeders Under 1,000 head feedlot capacity		Commercial Feedlots 1,000 head and over feedlot capacity		Total all Feedlots	
State	No. of Lots	Cattle Marketed 1,000 Head	No. of Lots	Cattle Marketed 1,000 Head	No. of Lots	Cattle Marketed 1,000 Head
Arizona	10	19	12	443	22	462
California	12	3	61	836	73	839
Colorado	130	70	170	2,200	300	2,270
Idaho	37	10	50	442	87	452
Illinois	9,160	725	40	90	9,200	815
Iowa	10,935	1,133	565	404	11,500	1,537
Kansas	1,636	70	264	4,125	1,900	4,195
Minnesota	6,441	404	59	96	6,500	500
Nebraska	9,050	1,340	450	3,340	9,500	4,680
Oklahoma	203	20	27	745	230	765
S. Dakota	4,146	224	54	381	4,200	605
Texas	852	90	148	5,170	1,000	5,260
Washington	65	10	15	446	80	456
13 States	42,661	4,094	1,931	18,742	44,592	22,836

The number of feedlots with 1,000 head or more capacity is the number of lots operating during the year. The number under 1,000 head capacity is the number at the end of the year.

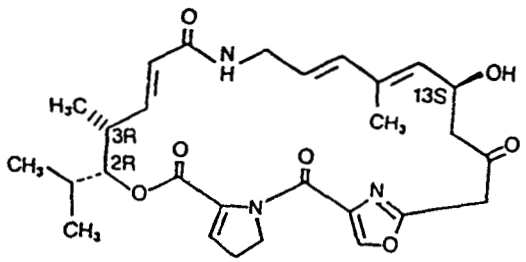
The type of environment present at and adjacent to cattle production facilities can include open fields, streams, ponds and woodlands, usually in a rural area. Approximately 65 to 70% of the cattle are raised in commercial feedlots and approximately 15 to 20% are raised on farmer feeders.(1)

5. IDENTIFICATION OF CHEMICAL SUBSTANCES: 000013

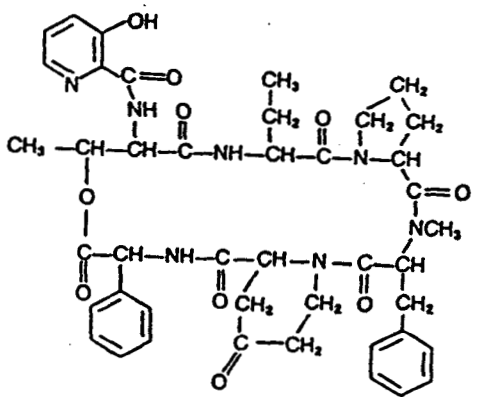
Virginiamycin (CAS-11006-76-1) is a composite antibiotic produced by **Streptomyces virginiae.**(2) It contains a mixture of two principal antibiotic components, virginiamycin Factor M and virginiamycin Factor S which act synergistically against a wide range of gram-positive organisms. Factor M is present in the largest concentration and is mainly active against **Staphylococcus aureus**, while Factor S is mainly active against **Bacillus subtilis.**

A. Structural Formulas:

Factor M:



Factor S:



B. Chemical/Physical Properties

Substance: Virginiamycin

Chemical Formula: Factor M - $C_{28}H_{35}N_3O_7$
Factor S - $C_{43}H_{49}N_7O_{10}$

Molecular Weights: Factor M - 525.607
Factor S - 823.91

Description: Amorphous, white powder

Melting Point: 138 - 140° (decomposition)

Solubility: Water solubility at pH 5.10 = 80.00 ppm
pH 7.26 = 77.08 ppm
pH 9.28 = 54.00 ppm (Appendix I)
Sparingly soluble in dilute acid and strong base. Soluble in methanol, ethanol, acetone, ethyl acetate, chloroform and benzene.

Stability: Rapid inactivation occurs in aqueous alkali above pH 9.5.

Dissociation Constant: Virginiamycin contains no ionizable functional groups that would dissociate under environmental conditions (Appendix II).

Octanol/Water Partitioning: K_{ow} at pH 5: 51.9-52.7, at pH 7: 54.1-54.6 and at pH 9: 30.3-32.3. A range of K_{ow} values are given since the coefficient was determined at two concentrations for each pH (Appendix III).

Ultraviolet-Visible Absorption Spectrum:

pH	Mean λ max (nm)	Mean Molar Absorptivity (L/mole*cm)
5	306.1	2067
5	353.2	1065
7	343.3	1323
9	338.2	3256

See Appendix IV for details.

6. INTRODUCTION OF SUBSTANCES INTO THE ENVIRONMENT:

Introduction of virginiamycin into the environment can occur from three sources, (a) the virginiamycin production facility, (b) the virginiamycin Type A Medicated Articles production facility and (c) the sites of intended use in cattle. A description of each source follows with calculations of the total virginiamycin entering the environment.

A. Description of the Virginiamycin Production Facility and Calculation of Environmental Exposure

Foreign Production

Virginiamycin is produced at SmithKline Beecham RIT, S.A., Rue de l'Institut 89, B- 1330, Rixensart, Belgium. General Environmental Control criteria are evaluated as part of a local governmental "General Plant Operating Permit" (86 page document) issued by the Province after consultation with the Ministry of Employment, the (Walloon) Ministry of Environment and the local community. The permit (Dossier #: 12/74679/26799) was issued on December 14, 1989 and is effective for 30 years. The permit was delivered by the Provincial Clerk by ordinance of the President of the Deputation in charge. The review covers noise levels, odors, water discharge and toxic emissions (if applicable).

Specific permits are issued to cover the following:

Waste Water Discharge Permit (12 page document): The permit (Dossier #: 93/ESu/AD-25091/00001) was issued on June 29, 1993 by the Minister in Charge at the Walloon Region Agency, Administration of the Environment and Natural Resources. The permit is valid for ten years from the date of issue and covers the maximum levels of surface and sewer discharge, in agreement with the existing convention between SmithKline Beecham Biologicals and the authorities operating the waste water treatment station, located at Religion Street, 1400, Nivelles, Belgium.

Well Pumping Permits (7 page documents): The permits (Dossier #s: 1992/2/c/50044 (P2), 1992/2/c/50043 (P3) and 1992/2/c/50042 (P4) were issued on January 13, 1993 by the Minister in Charge of the Ministry of Environment for Walloon Region. The permits are valid for five years from the date of issue. The permits allow a maximum volume of 1,000,000 m^3 of water to be pumped per well per year.

Specific environmental control practices utilized at the plant are:

i) Hazardous Liquid Waste Stream

Spent solvent streams generated in the fermentation process are recovered and recycled for process reuse. Residue recovery still bottoms are sent to the spent broth storage tank for processing. The organic stream is a closed system with no direct discharge to the environment.

ii) Liquid Waste Stream

Spent Broth:

Non-volatile residues from recovery are combined and classified as spent broth. The broth is sent to a reverse osmosis unit for concentration.

A waste stream from the concentration step which contains no virginiamycin is discharged to the municipal sewer. As a result of the approval of virginiamycin for cattle in the United States, approximately 140 additional tons of concentrated spent broth will be disposed of per year.

Excess Spent Broth:

This additional spent broth will be disposed of by incineration in compliance with our "General Plant Operating Permit".

Discharge to a local municipal waste treatment system for biological treatment (under permit). None of the additional 140 tons of concentrated spent broth will be discharged into the municipal waste treatment system.

Other Waste Streams:

Waste streams are generated from other manufacturing processes:

The scrubber blowdown containing no virginiamycin is discharged with non-contact cooling water to the Lasne River by permit of the National Ministry of Health (1976).

The other wastes are blended with the Antibiotic Plant discharges and monitored for flow volume, pH, biological oxygen demand, chemical oxygen demand and detergents, during discharge to the municipal sewer.

These discharges are controlled by agreement with IWB (Intercommuale du Brabant Walloon) municipal Sewer Plant and are in compliance with our "General Plant Operating Permit".

iii) Air Emissions

Vapors:

Air emissions are scrubbed in a packed column, with blowdown from the system discharged to the river as it contains no active virginiamycin. These vents are not regulated by any formal government regulation but incorporate a "state-of-the-art" treatment system.

Solvent storage tanks are equipped with flame arrestors.

Dust:

No significant amount of dust escapes into the environment since dust from the fermentation process is disposed of in the scrubber blowdown waste discharge.

All dust from conveying dry feed systems is vented through a bag filter and the collected solids are reprocessed.

iv) Dry Solid Wastes:

All solid waste materials are disposed of by a contractor in compliance with local and national laws.

v) Calculation of environmental exposures:

All 140 additional tons of concentrated spent broth per year will be disposed of by incineration in compliance with our "General Plant Operating Permit" and will not constitute any additional environmental exposure.

By signing this environmental assessment, the SmithKline Beecham Animal Health representative certifies compliance with the cited emissions requirements at SmithKline RIT.

vi. Occupational Controls

(1) Introduction

SmithKline Beecham biologicals in Rixensart, Belgium, has an occupational hygiene and safety policy in compliance with the SB Corporate Occupational Health & Safety Standards, issued by the SB company, and the Belgian General Regulation for Work Protection, issued by the Belgian Ministry of Employment.

(2) Regulations

The corporate Occupational Health & Safety Standards states the general safety, occupational hygiene and environmental standards for all sites part of SB corporate.

The Belgian General Regulation for Work Protection, so-called "Reglement General pour la Protection du Travail" and frequently called "RGPT" is the compendium of laws to be respected by all professional groups and activities in the Belgian State, and takes precedence to the corporate standards when applicable. It states the provisions relating to general security, machinery, tools & equipment, movements, fire protection, transportation lettering & signs, working place ambiance, clothing, medical survey & assistance, products safety.

(3) Organization to the Site

In pursuance of the article 103 of the RGPT, safety and hygiene are controlled by means of a security service that employs 14 people and a medical service that employs one Doctor in Medicine plus 2 people, both supervised by a Committee for Security and Hygiene. The security service writes the safety sheets, security procedures, organizes a safety improvement plan with a weekly meeting and a yearly report, organizes internal inspections with reporting, and coordinates Federal or Corporate inspections.

The Medical Service organizes a medical check-up for all the people employed on the site of production, twice a year for the people in production, maintenance and research, and once a year for the others. A medical record is kept for each individual, and a synthesis report is written every year.

The Committee for Safety and Hygiene meets on a monthly basis with representatives of: the security service, the medical service, the blueworkers, the employees and the management. The proceedings of these meetings are made available monthly.

Besides these legal provisions, the Antibiotic Plant also employs a Safety Adviser who writes safety procedures and organizes safety inspections in all the services. SB Corporate also organizes a complete blood analysis for all the members of the company on a yearly basis. A Biosafety Committee evaluates the danger of the manipulation of all the strains used on the site.

Available Documents:

- Monthly reports of the Committee for Security and Hygiene
- Yearly reports of the Medical Service
- Safety sheets
- Safety procedures
- Inspection reports
- Personnel medical records
- Yearly security plan report

B. Description of Virginiamycin Type A Medicated Articles Production Facility and Calculation of Environmental Exposure

V-Max™ Type A Medicated Articles are manufactured at our facility at 4444 South 76th Street, Omaha, Nebraska 68127. Environmental control practices adhered to are:

i) Liquid Waste Stream

Waste liquids are generated from the manufacturing of V-Max™ Type A Medicated Articles as a result of washing the empty production equipment after use and small quantities from the analytical laboratory. This liquid waste contains very small

amounts of virginiamycin and inert carrier. This waste is regulated by the City of Omaha Municipal Code Chapter 31.

Treatment of this waste by the City of Omaha Waste Water System, NPDES Permit No. NE0036358, is regulated by the Nebraska Department of Environmental Control under the Nebraska Environmental Protection Act, Chapter 81, Article 15 as subject to regulations under Section 307b of the Federal Water Pollution Control Act, 40 CFR Part 439. This liquid waste requires no pretreatment and is in compliance with the above referenced laws and regulations.

ii) Air Emissions

Air emissions from the production of V-Max™ Type A Medicated Articles that escape our production system consist of dust which contains virginiamycin and the inert carrier. Only negligible amounts of this dust escape outside the plant. The dust is contained inside the plant by keeping the manufacturing system closed as much as possible and using the central dust collector (MAC Model 72AV25, bag filter, 25,000 CFM) to extract dust that escapes the system. The dust collected by the system is deposited in a central container and is disposed of at the City of Omaha County landfill by the Tecrep Company.

This system meets the requirements of the Nebraska Department of Environmental Control, as prescribed under the Nebraska Environmental Protection Act, Chapter 81, Article 15. Air emissions associated with the production of V-Max™ Type A Medicated Articles contain no hazardous materials regulated by the State of Nebraska; therefore, the state does not require a permit.

iii) Dry Solid Waste

Dry solid waste is disposed of at the City of Omaha County landfill by Browning - Ferris Industries Waste Systems. This municipal landfill is regulated by the Nebraska Department of Environmental Control. The additional dry solid waste which includes flush material, floor sweepings, dust from the dust collector (approximately 200 lbs/year) and outdated and returned goods (approximately 200 lbs/year) which contain approximately 40 lbs of virginiamycin activity, will be disposed of in accordance with the above referenced laws and regulations.

iv) Employee Protection

Material Safety Data Sheets are available for employees who work in the production area, (Appendix V). In addition, employees in the production and packaging areas wear protective clothing and dust respirators as needed, to assure compliance with OSHA standards, CFR 29, Part 1900 to 1910 and OSHA's Hazard Communication, CFR 29, Part 1910. Employee training and industrial hygiene programs are routine plant operations.

v) Calculation of Environmental Exposure

The additional amount of virginiamycin contained in liquid waste is negligible. Approximately 200 lbs/year of dust and approximately 200 lbs/year of returned goods disposed of at the City of Omaha landfill amount to a maximum of approximately 40 lbs (18 kg) of virginiamycin waste at this facility on a yearly basis.

By signing this environmental assessment, the SmithKline Beecham Animal Health representative certifies that the Type A Medicated Articles production facility complies with the cited emissions requirements.

C. Calculation of Environmental Exposure Due to Use in Cattle

In cattle as in poultry, (chickens and turkeys) virginiamycin residues are excreted (75 to 94%) mainly via the feces (Appendix XIV).[3] Therefore, the stability of virginiamycin in manure is the major determining factor of environmental impact.

Environmental fate studies were conducted with chicken excreta and swine feces and reported previously in NADA 91-467, February 1, 1979, to support the approval, March 27, 1981, for use of virginiamycin in broiler chickens and July 25, 1988 in turkeys. Additional fortification studies were similarly conducted with cattle manure to demonstrate that comparable results are attained regardless of the origin of the fecal matrix. This information is discussed in Section 7 of this assessment to support the environmental considerations for use of virginiamycin in cattle since any effect on the environment would be similar.

Although the patterns of excretion of virginiamycin residues in cattle and poultry were similar, the residue profiles would be qualitatively different. In a study entitled "Virginiamycin Metabolism in Cattle Rumen Fluid" (Appendix VI) rapid metabolism of factor M to several metabolites with less microbial activity than the parent was evident. Factor S remained unchanged. The antimicrobial activity of virginiamycin decreased rapidly ($t\ 1/2 \simeq 8$ hours). The metabolism is discussed further in Section 7.A of this assessment.

Decisions regarding the environmental safety of virginiamycin are based on the relationship between the virginiamycin residue concentration expected in the environment and residue levels expected to have no adverse effect on aquatic and terrestrial resources based on appropriate environmental effects screening tests. Environmental residues are estimated from drug use and properties governing the behavior of the excreted residues in the commercial environment and when spread as fertilizer onto agricultural soils.

i) Theoretical Amounts of Virginiamycin Eliminated by Target Animals

The following information will be used to calculate the quantity and concentration of virginiamycin that could possibly enter the environment assuming commercial feedlot or farmer feeder cattle

received virginiamycin at the maximum dose level of 25 g/ton for 150 days.

It is estimated that approximately 25 to 30 additional tons of virginiamycin per year will be administered to cattle. The major use of virginiamycin will be in feedlot cattle, hence, the "worst case" environmental concentrations and exposures to virginiamycin residues are expected to occur when the drug is used in feedlot cattle. As a consequence, residues of virginiamycin will be introduced into agricultural soils, since feedlot manure is introduced into this environment as a fertilizer. In order to determine the expected concentrations of virginiamycin residues in feedlot and agricultural soils and how they relate to the environmental studies, the following information was considered:

Maximum length of medication	150 days
Maximum approved usage level (100% dry matter basis)	25 g/ton (27.8 mg/kg)
Average daily feed consumption per head	9 kg(4)
Expected days to finish for feedlot steers and heifers	136 days(5)
Maximum expected spreading rate of manure onto agricultural soil	20 tons per acre(6)
Manure excreted per 1000 lb live weight for finishing cattle	8.5 tons/year(6)
Manure excreted per 1,000 lb live weight adjusted for 60% loss of moisture	3.4 tons/year(6)

Virginiamycin will be administered at a maximum estimated dose level of 25 g/ton in feed which is equivalent to 27.8 mg/kg feed. The only expected area of concentrated use for the product would be in feedlots. Calculations performed to estimate the expected residues of virginiamycin as runoff from the feedlot and in agricultural soils follow.

27.8 mg/kg feed x 9 kg feed/head/day = 250 mg Vm/head/day and 250 mg Vm/head/day x 150 days maximum length of medication
= 37,500 mg virginiamycin per animal

or

37.5 g virginiamycin per animal

Excretion and tissue depletion studies in beef cattle dosed with ^{14}C-virginiamycin demonstrated that an average of 88% of the radioactivity

was excreted in the urine and feces within 7 days after administration of the dose.[3] The calculation presented above is based on 100% excretion of virginiamycin residues, thus presenting the 'worst case' scenario.

Since, on the average, manure is not removed from the feedlot more than once per feedout period, the expected concentration of virginiamycin residues can be calculated in feedlot manure. Virginiamycin residue concentrations are based on complete elimination of the virginiamycin dose and a uniform mixing of the dose into the total amount of manure eliminated during the feedout period. In addition, the total amount of virginiamycin residues eliminated in the average feedlot per animal per feedout period is calculated as follows:

150 days x 3.4 tons/365 days = 1.3973 tons * manure/animal/medication period

*This figure is based on a 1,000 lb animal for a 150 day period.

The maximum estimated concentration of virginiamycin residues in the manure would then be:

37,500 mg virginiamycin/1.3973 tons manure = 26,837 mg Vm/ton manure or 13 mg/lb manure which is equivalent to approximately 30 mg/kg or 30 ppm in cattle manure

ii) Theoretical Amounts of Virginiamycin Residues in Feedlot Runoff and in Agricultural Soil Under Two Extreme Conditions

a. Feedlot Runoff
Under "worst case" conditions assuming all of the applied virginiamycin residues are contained in a two inch rainfall in the feedlot where each animal occupies 200 square feet of space[11], the concentration of these residues in the runoff (without considering biodegradation or sorption effects) can be estimated as follows:

$$\frac{200\ ft^2}{animal} \times 2''\ rainfall \times \frac{1\ ft}{12''} \times \frac{28.317\ L}{ft^3} = 943.9\ Liters\ Water$$

$$\frac{37{,}500\ mg\ Vm/animal}{943.9\ L\ water} = 39.729\ mg\ Vm/L\ water$$

or

39.729 ppm virginiamycin

b. Agricultural Soil
If cattle receive virginiamycin at the highest use level, 25 g/ton (approximately 250 mg/head/day), the total amount of virginiamycin consumed per animal for the maximum length of medication (150 days) is 37.5 g as calculated in Section 6. Since

65-70% of the cattle are raised in commercial feedlots it is reasonable to assume that evaluation of this source of introduction of virginiamycin to the environment would represent the "worst case" scenario.

The maximum expected spreading rate of cattle manure per acre of agricultural soil was estimated to be 20 tons per acre. Since 20 tons of manure equals 40,000 lb and the maximum estimated concentration of virginiamycin per pound of manure is 13 mg, the concentration of virginiamycin in fertilized soil can be calculated as follows:

40,000 lb manure/acre x 13 mg Vm/lb manure = 520,000 mg Vm per acre

If it is assumed that manure will be mixed into the top six inches of soil, this is equivalent to a soil weight of 9.25×10^5 kg/acre (1.5g/cc)[8]. Therefore:

520,000 mg Vm per acre / 9.25×10^5 kg soil per acre = 0.56 mg virginiamycin/kg soil or 0.56 ppm in soil

This maximum concentration of 0.56 ppm does not reflect any loss of parent compound activity due to metabolic transformations in rumen fluid, biodegradation effects in feces and soil and probably in water.

7. FATE OF EMITTED SUBSTANCES IN THE ENVIRONMENT

As previously mentioned, the compound's major route of entry into the environment will be through fecal droppings. However, the possible impact on the environment due to its usage in cattle is minimal as very little of the compound has a tendency to accumulate. The evidence for minimal persistence can be found from the studies described below (Sections 7. A, B and C).

A. Decrease of antimicrobial activity of virginiamycin in cattle rumen fluid in vitro:

In a screening experiment (Appendix VI), rumen fluid collected from a fistulated steer was fortified with ^{14}C-virginiamycin and incubated at 39°C for up to 48 hours. Four metabolites were isolated and identified by MS and NMR techniques. All of the isolated metabolites were derived from the reduction of factor M; factor S was not metabolized in the rumen fluid. One metabolite demonstrated significant anti-microbial activity, two were devoid of activity and one was not tested. Overall anti-microbial activity decreased rapidly with an initial half-life of approximately 8 hours. Since the activity of the two factors is nearly seven-fold that of either separately, the production of inactive metabolites is consistent with the observed loss of anti-microbial activity.

B. Disappearance of virginiamycin activity in cattle manure

As mentioned in Section 6, the environmental exposure from use of virginiamycin in cattle is through manure applied as fertilizer to agricultural soil and the extent of exposure is related to the stability of virginiamycin in the environment. In a screening study, the stability of virginiamycin in cattle manure was evaluated since previous studies on the stability of virginiamycin in swine manure and poultry droppings demonstrated that virginiamycin undergoes rapid and extensive potency loss (89-94% loss in 14 days when measured against **Corynebacterium xerosis**) in both of these fecal matrices. Manure from non-medicated cattle was fortified with virginiamycin at a level of 34.8 ppm and the biological potency measured after 0,1,3,5,7,10 and 14 days. Within 7 days, only 13.2% (4.6 ppm) of the initial level of microbiological activity remained. Microbiological activity was below the lowest point of the standard curve (0.64 mg/mL) by day 10. The calculated half-life of virginiamycin potency in cattle feces was 2.5 days (Appendix VII).

C. Aerobic biodegradation of virginiamycin in soil

In an experiment using radiolabelled ^{14}C-virginiamycin (Appendix IX), the biodegradation potential of the compound in a variety of soils was measured for 64 days. In addition to determining the cumulative percentages of $^{14}CO_2$ evolved after 64 days, high performance liquid chromatographic (HPLC) analysis for parent virginiamycin was performed after extracting soil samples from days 1 and 64. While the mean cumulative percentage of $^{14}CO_2$ evolved after 64 days from the soils dosed with virginiamycin were 20.5, 17.6 and 12.4% for three soil types, Kansas, Ohio and Mississippi, respectively, the HPLC results indicated that virginiamycin is extensively degraded in soil into a series of minor components with no single degradation product present at >10% of the initial concentration. The results are given in Table II.

Table II

Results of the HPLC Analysis of Soil Extracts

	Day 7		Day 64	
Soil Type	Factor M % of nominal	Factor S % of nominal	Factor M % of nominal	Factor S % of nominal
Kansas Silty Clay Loam	70.4	74.7	<6.4 <6.3	<6.5 8.6
Ohio Clay Loam	96.8	84.7	<6.3 <6.4	<6.4 <6.4
Mississippi SiltyClay Loam	83.3	84.4	<6.2 <6.2	<6.3 <6.3

In a separate non-definitive experiment (Appendix VIII), aerobic biodegradation of ^{14}C-virginiamycin was examined following a protocol similar to one used above. The cumulative percentage of $^{14}CO_2$ evolved after 64 days was determined. ^{14}C-Virginiamycin appeared to biodegrade in 3 different soil types. The degradation proceeded in all soils with no apparent lag period. The calculated half-lives ranged from 87 to 173 days for the three soils tested, with first order rate constants of 0.004 to 0.008 $day.^{-1}$ A summary of $^{14}CO_2$ production, ^{14}C-volatile organics production and mass balance over time are presented for ^{14}C virginiamycin and a reference standard, ^{14}C-glucose, in Table III.

TABLE III

Cumulative Production of $^{14}CO_2$ and ^{14}C-Volatile Organics Expressed as Percent of Initial DPM Added (Average of Replicates)

Treatment	Soil+ Type	Average % $^{14}CO_2$	Non-CO_2	Mass Balance
^{14}C-Virginiamycin	CA	40	1	90
^{14}C-Glucose	CA	46	1	93
^{14}C-Virginiamycin	CF	30	2	92
^{14}C-Glucose	CF	66	0	100
^{14}C-Virginiamycin	PM	25	1	87
^{14}C-Glucose	PM	58	1	93

+ Soil characteristics are given in Appendix VIII.

As presented in Section 6.C, the maximum exposure levels possible from virginiamycin to soil (through manure applied as fertilizer) was calculated to be 0.56 ppm. From the observed rate of biodegradation in soil, this concentration can be expected to decrease fairly rapidly in soil.

The rate of mineralization of virginiamycin is slower than the reference chemical in both studies. This behavior is to be expected as the structures of factors M and S of virginiamycin is more complex than the reference glucose and the C content of the virginiamycin (average 64%) is higher than the latter. However, the tranformation of the chemical into a series of minor components other than $^{14}CO_2$ during the study indicated that biodegradation is a major pathway of degradation. Therefore, the studies above demonstrate that any levels of virginiamycin remaining in the manure applied to soil via fertilization, would be reduced through biodegradation.

D. Octanol/water partitioning of virginiamycin

An octanol/water partitioning study was performed in order to evaluate the potential for virginiamycin absorption in animals and plants. The partition coefficient (K_{ow}) of virginiamycin was determined at 25° ± 1°C in pH 5, 7 and 9 buffered solutions. At each pH, the K_{ow} was determined at three different concentrations of the test substance (Appendix III). The results are summarized in Table IV. With log K_{ow} of less than 2, virginiamycin is expected to be moderately lipophilic and have lower bioconcentration potentials than erythromycin (log K_{ow} = 2.48). Bioconcentration is also governed by lipid and protein content of the organism[12]. Virginiamycin with its hydrogen bonding groups on both factors M and S is expected to be bound to protein more strongly than to lipid. Additional factors that may hinder its bioconcentration would be its large molecular cross section and metabolic transformation. When virginiamycin was fed to chickens for five days at a rate of 20 g/ton, no significant blood levels could be detected, indicating poor absorption. Low tissue residues were also observed in cattle and laboratory animals as well as in poultry and swine, further supporting this poor absorption.

Table IV
Octanol/Water Partition Coefficient of ^{14}C-Virginiamycin

pH	Concentration (µg/mL)	Average K_{ow}
5	4.0, 7.3	51.9 ± 1.4*
	53	52.7 ± 1.7
7	4.0, 7.3	54.1 ± 5.1*
	53	54.6 ± 2.4
9	4.0, 7.3	32.3 ± 1.4*
	53	30.3 ± 1.0

*K_{ow} values for concentrations 4.0 and 7.3 µg/mL were averaged.

E. Soil Adsorption/Desorption

Soil adsorption/desorption studies (Appendix X) were conducted to determine the rate and extent of adsorption/desorption of virginiamycin to soil. The study design consisted of three phases: preliminary/screening, kinetics and isotherm experiments. Testing was performed using three soils varying in pH, organic matter and cation exchange capacity, to determine the adsorption coefficients (K_d) and adsorption coefficients based upon the organic carbon content (K_{oc}) of the soil at equilibrium. A summary of these data is presented in Table V.

Table V

Summary of Adsorption Coefficients for ^{14}C Virginiamcyin

	pH	% Adsorbed	Std Dev	Estimated K_d	Std Dev	Est K_{oc}	Std Dev
Soil #1*	5.3	57.9	1.9	6.9	0.5	683	50
Soil #2*	7.2	68.4	0.9	10.8	0.4	509	19
Soil #3*	6.9	72.7	0.2	13.3	0.1	675	5

* Characteristics of experimental soils are given in Appendix X.

In comparison with the ranking of chemicals with known mobility, based upon the K_{oc} values, virginiamycin can be ranked between moderately and tightly bound to soil.

F. Prediction of environmental concentrations of and exposure to virginiamycin entering the environment

i) Air

Virginiamycin is not volatile, therefore the only exposure to air would be in the form of dust at production sites. However, this exposure is minimized by the use of a filter system and would not be expected to have any effect on the environment as discussed in Section 6.

ii) Freshwater, estuarine and marine ecosystems

Any exposure of virginiamycin to water would occur from settlement of dust from production sites onto the ground with subsequent possible run off into waterways. Additionally, manure from cattle raised on commercial feedlots would be a direct source of exposure into the environment which could potentially run off into waterways. However, considering its potential to adsorb to solid particles and its microbial degradation, the likelihood of the exposure of aquatic organisms to toxic concentrations of virginiamycin from runoff will be small.

The distribution adsorption coefficient, K_d, which is the ratio of a test chemical sorbed to that in solution at equilibrium can be used in a non-definitive fashion to estimate the maximum runoff concentrations for virginiamycin (Appendix XIII). Since this is not a validated algorithm, the data deriving from it should be treated as qualitative.

4247.55 kg soil x 6.9 X = mass of Vm in soil
943.9L water x 1 X = mass of Vm in water

(4247.55 x 6.9 X) + (943.9 x 1 X) = 30,252.00 X

30,252.00 X = 37,500 mg Vm (total Vm available per animal)

X = 1.24 mg/L = concentration of Vm in runoff water

where:

4247.55 kg soil = calculated area of exposure
6.9 = lowest K_d value of 3 soil types tested
943.9 L water = calculated volume of rainfall to the area of exposure

Again, this value is only an estimate and has not been adjusted for biodegradation effects.

iii) Terrestrial ecosystems

The practice of applying livestock manure to fertilize agricultural soil, necessitates an assessment of the maximum concentration of excreted virginiamycin in the soil.

Using the proposed maximum dosage in cattle for the entire 150 days of treatment, the maximum estimated fecal concentration is 30 ppm. (This level is below the maximum fecal concentration reported in the environmental impact analysis report for approval of virginiamycin use in swine - NADA submission March 15, 1978, approval September 19, 1978.)

Application of the cattle feces to soil would result in a maximum concentration of 0.56 mg Vm/kg soil or 0.56 ppm in soil.

The potential for mineralization of virginiamycin in soil (Section 7.C) ranged from 12.4% to 20.5% within 64 days. However, extensive degradation of Vm into a series of minor components occurred within 64 days with no single degradation product present at >10% of the initial concentration.

Assuming no reduction of Vm is occurring in the manure prior to spreading, mineralization by soil microbes alone would reduce the concentration of virginiamycin from 0.56 ppm to levels ranging from 0.34 to 0.42 ppm in the soil within 64 days. Additionally, >90% of the parent is broken down into minor components other than CO_2 and H_2O within this period.

As discussed above, the concentration of virginiamycin to which the terrestrial ecosystems would be exposed is negligible. Since the product suffers degradation in feces and soil, the opportunity for accumulation to occur in the environment is unlikely, thereby eliminating a potential environmental concern from build-up to an inhibitory concentration against terrestrial organisms.

8. ENVIRONMENTAL EFFECTS OF RELEASED SUBSTANCES

Virginiamycin is classified as a narrow spectrum antibiotic primarily active against gram-positive bacteria and not used in human medicine in this hemisphere. Virginiamycin has met the human and animal safety criteria for antibiotics in animal feeds and its use does not constitute a risk or a human health hazard (Appendix V).

A. Occupational Safety Studies

The following studies were performed in order to determine the safety of virginiamycin residues, with the resulting no-observed-effect levels (NOEL). Details of each study are contained in the Freedom of Information Summaries for Use of Virginiamycin in Broilers and Turkeys. The results of these studies can also be used as guidance for occupational exposure.

Virginiamycin is non-toxic. No toxic effect attributable to virginiamycin could be demonstrated in any of the following acute and chronic toxicity studies performed on a variety of animals including mice, rats, dogs, turkeys, chickens and cattle.

i) Target Animal Safety in Cattle - Cattle administered feed containing virginiamycin at 25, 75, 125 or 625 g/ton exhibited no significant adverse health or toxicological effects.

ii) Safety in Broilers - Broilers fed 2000 ppm of virginiamycin in feed for 24 hours, then observed for 14 days, exhibited no evidence of toxicity. Broilers fed 22, 66 and 110 ppm of virginiamycin in feed for seven weeks, exhibited no adverse effects which could be attributed to virginiamycin.

iii) Safety in Turkeys - Turkeys were fed 0, 20, 100 and 200g of virginiamycin per ton of feed from one day of age until 16 weeks and 20 weeks for hens and toms, respectively, without any significant adverse effect.

iv)

STUDY	NOEL
Acute Oral Toxicity in Mice	1500 mg/kg
Subacute Toxicity in Rats	100 mg/kg
Subacute Toxicity in Dogs	100 mg/kg
Teratology in Rats	75 mg/kg
Teratology in Mice	1000 mg/kg
Subchronic Toxicity in Beagle Dogs	25 mg/kg
Two Generation Reproduction in Rats	100 mg/kg
Chronic Toxicity (Carcinogenicity) in Rats	50 mg/kg
Chronic Toxicity (Carcinogenicity) in Mice	1000 mg/kg

B. Environmental Effect Studies:

i) Phytotoxicity study

Litter from chickens consuming virginiamycin medicated feed (20 g/ton) was applied to loam soil in greenhouse flats at a rate of 4-10 tons per acre. These flats, and others containing untreated loam or application of litter from non-medicated chickens (120 total flats) were planted with wheat, barley, fescue, peppers, tomatoes or corn.

At the termination of the study, there was some inhibition of the growth of beans and cucumber noted. There was 15% reduction in the number of beans and cucumber plants compared to controls at 4 tons per acre treatment. At 5 tons per acre application, there was 20% reduction of cucumber plants relative to controls. However, there were no phytotoxic symptoms observed on any of the plants.

ii) Fish toxicity studies

Rainbow trout and bluegill sunfish were exposed to virginiamycin treated water for periods of 24, 48 or 96 hours. Toxicity was evaluated in terms of the concentration of drug which produced 50% mortality (LC_{50}). The test showed that extremely high concentrations of virginiamycin (more than 225 ppm), were required to produce 50% mortality in either type of fish (Appendix XII).

C. Potential Impact on the Environment

i) Air

As discussed in Section 7, virginiamycin would not present any adverse effect on the environment since it is non-volatile. Additionally, any dust from production facilities is minimal due to filtration and the negligible amount that may escape filtration would only result in exposure to the soil or water where the activity rapidly disappears negating any potential impact on the environment.

ii) Fresh water, estuarine, and marine ecosystems

Without considering biodegradation effects, the theoretical "worst case" concentration of virginiamycin in agricultural runoff was estimated to be 39.729 mg/L (See Section 6.C.ii.a). Even at this concentration, there are sufficient safety margins to aquatic species such as blue gill and rainbow trout (LC_{50} 225-338 mg/L). However, the runoff value is expected to be impacted by the adsorption of the compound to soil particulates (see Section 7.F.ii.) and dilution at the water body. Biodegradation is also expected to continue in water, thereby, reducing the exposure concentration further. Based on Vm water solubility (54-80 mg/L at pH 9-5), the water body is not the preferred compartment for its distribution. Its tendency to absorb light from the visible region of the spectrum

indicates a possible photolytic pathway of degradation. Additionally, some hydrolysis of Vm was evident at pH 7 and 9 during the conduct of the time-dependent water solubility study (Appendix I). Therefore, the exposure of aquatic species to Vm from runoff is not expected to cause any adverse effects.

iii) Terrestrial ecosystems

The major exposure of virginiamycin to the terrestrial ecosystem is through cattle manure spread onto soil. Due to the disappearance of virginiamycin with time as a result of biodegradation (in soil and in feces), expected concentration in agricultural soil would be negligible. The amount of exposure, as calculated in Section 7, would be decreased with time in soil, with no adverse effects to the animals, plants and soil microbes tested. Any uptake by plants would be negligible for similar reasons, and the results of the phytotoxicity studies in plants discussed in Section 8, demonstrated that virginiamycin in poultry fecal material applied to soil had no significant phytotoxic effect on plants except for some retardation of growth in some species which may be a transient effect. The low absorption of virginiamycin in cattle, as well as in broilers, turkeys and swine (as determined from tissue residue studies) suggests that similar absorption patterns would occur in other vertebrate animals if they ingested the antibiotic[3,9].

Listed below are a number of microbes indigenous to soil, and M.I.C. values for virginiamycin. Considering the data in light of possible soil concentration and eventual disappearance of virginiamycin activity, it is inconceivable that soil levels of virginiamycin would ever approach the M.I.C.s listed below.

In Vitro Minimal Inhibitory Concentrations (M.I.C.)

ORGANISM	M.I.C. OF VIRGINIAMYCIN µg/ml (ppm)[7]
Mycoplana bullata ATCC 4279	20
Mycoplana dimorpha ATCC 4279	100
Hydrogenomonas sp.	100
Citrobacter sp. 1	100
Citrobacter sp. 2	1000
Flavobacterium sp.	1000
Klebsiella sp.	1000
Thiobacillus thiooxydans 504 DSM	10
Cythophaga johnsonae 425 DSM	10
Rhodopseudomonas sp.	1000
Hyphomicrobium sp.	100
Rhodospseudomonas sphaeroides 158 DSM	100
Nitrobacter sp.	1000

Virginiamycin may be categorized primarily as a gram-positive antibiotic. Based on its in vitro spectrum of activity, it exhibits neither anti-fungal nor gram-negative activity. This immediately eliminates any potential impact on those organisms, the gram-

negatives, which play a major role in the decomposition of organic matter in the soil and nutrient recycling in the soil. For example, the major soil reactions are all accomplished by gram-negative organisms such as **Nitrosomonas, Nitrobacter** and **Thiobacillus.**

Other gram-negative organisms are important in the process of non-symbiotic and symbiotic nitrogen fixation. The genus **Azotobacter** is responsible for non-symbiotic nitrogen fixation, while that of **Rhizobium** is responsible for nodulation and symbiotic nitrogen fixation. By virtue of the gram-negative origin of all of these soil reactions, virginiamycin residues should have no impact on normal soil microbiota because, a) it is exclusively a gram-positive antibiotic, and b) only very low concentrations can be expected in the soil under approved conditions of use.

Some gram-positive anaerobes, such as the **Clostridia,** play some role in the fixation of nitrogen. These organisms are more acid tolerant than aerobes and perhaps for that reason are more widespread. Under suitable conditions, they are capable of nitrogen fixation.

Regarding the gram-positive anaerobes, the minimal inhibitory concentration (M.I.C.) of virginiamycin against **Clostridium welchii,** is 0.5 μg/ml which is well above the maximum estimated soil concentrations previously presented.

Another important factor to be considered regarding the effect of virginiamycin residues on soil microorganisms is the impact on soil yeast and fungi. Again, a review of the <u>in vitro</u> spectrum leads one to conclude an absence of significant impact, based on no activity against the yeast **Candida albicans,** and fungus **Trichophyton mentagrophytes** 8410.

As the degradation of virginiamycin in soil is continuing, there can be no opportunity for accumulation in the environment, thereby eliminating the possibility for build-up to an inhibitory concentration against anaerobes.

Virginiamycin has a combination of features which distinguish it from many antibacterial agents. It exhibits the feature of bacteriopause; i.e., bacteria which come into contact with virginiamycin for a short time lose their ability to multiply for a considerable time after withdrawal of the product. It is bactericidal, acting primarily on gram-positive organisms through its ability to inhibit protein synthesis. Although its mode of action is not completely understood, evidence supports the theory that virginiamycin binds to an acceptor site on the ribosomal subunit thus interfering with peptide chain formation. This binding is irreversible and probably accounts for the bactericidal nature of its activity.

Total antibiotic activity of virginiamycin depends on synergistic interaction between its two component factors (M & S). Each factor has a different spectrum of activity. For example, Factor M

is active against both Micrococci (Staphylococci and Streptococci), but the combination of the two factors is far stronger in activity. Against **Corynebacterium xerosis**, Factor M alone has a Minimum Inhibitory Concentration (M.I.C.) of 0.2 mg/ml, while the M.I.C. of virginiamycin against **C. xerosis** is 0.03 mg/ml(2). The activity of Factor M is undoubtedly potentiated by the presence of Factor S, although Factor S alone has little or no activity against **C. xerosis**. Thus, the activity of the two factors together is nearly seven-fold that of either separately.

Virginiamycin has already met the Human and Animal Health Safety Criteria for Antibiotics in Animal Feeds.

The following table lists the M.I.Cs. of virginiamycin against a variety of bacterial organisms.

In vitro Minimal Inhibitory Concentrations (M.I.C.) in mg/ml(2,10)

Organism	M.I.C. of virginiamycin
Staphylococcus aureus	0.2
Sarcina lutea	0.03
Streptococcus pneumoniae	0.07
Streptococcus faecalis	15
Corynebacterium xerosis	0.03
Hemophilus pertussis	0.4
Neisseria meningitidis	0.1
Clostridium welchii	0.5
Bacillus subtilis	0.04
Lactobacillus acidophilus	0.5
Escherichia coli	>100
Proteus mirabilis	>100
Pasteurella pestis	3
Shigella flexneri	>100
Brucella abortus	75
Mycobacterium tuberculosis	1
Candida albicans	>100
Trichomonas vaginalis	>100
Mycoplasma gallisepticum	0.05
Leptospirae	0.002
Treponema hyodysenteriae	0.65

There are no known adverse environmental effects. Potential pollutants resulting from the manufacturing process are contained within the manufacturing facility and are disposed of in compliance with provincial, federal, state and local regulations. The compound is excreted in very low concentrations as the intact drug even after administration at the highest recommended use level for prolonged periods. Owing to its microbial degradation pathway, the persistence of this compound in the environment is not likely. It is only sparingly soluble in water; thus, the possibility of water contamination by leaching or from runoff is not a major pathway of distribution between environmental compartments. Therefore, no further measures are necessary to

mitigate or avoid potential adverse environmental impacts since no potential adverse effects are apparent.

9. USE OF RESOURCES AND ENERGY:

Minimal natural resources and energy are required to transport, use and dispose of waste resulting from the production of virginiamycin or from its use as a feed additive. The transportation to a landfill of any excess fermentation waste is minimized and disposal is performed in accordance with national, state and local requirements. No additional resources are required to remove waste containing virginiamycin from use in cattle since manure is removed from these production facilities regardless of additive use. Energy resources (gas, oil, electricity, etc.) are non-recoverable resources and are considered normal requirements of manufacturing/ production.

Virginiamycin has no known effects on endangered or threatened species or on property listed in or eligible for listing in The National Register of Historic Places.

Since virginiamycin is produced by a bacterial fermentation process, the expenditure of manufacturing resources is minimal and approximately 99% of the solvents used are recovered and recycled. Hence, no significant commitment of irretrievable resources will result from the production of virginiamycin.

10. MITIGATION MEASURES

Material Safety Data Sheets, Appendix V, are available for employees who work in the production area. In addition, employees in the production and packaging areas wear protective clothing and dust respirators as needed, to assure compliance with OSHA standards as discussed in Section 6. No other mitigation measures are necessary since virginiamycin is non-toxic and does not pose any known harm to the environment.

11. ALTERNATIVE TO THE PROPOSED ACTION:

No potential environmental impacts are apparent for the use of virginiamycin in cattle or for currently approved species (swine, broilers and turkeys). The only specific alternative to the proposed actions would be refusal to approve the New Animal Drug Application. This would, however, deny the producer the benefits which could be realized by use of virginiamycin in terms of the economic gain afforded by increased weight gain, improved feed efficiency and reduced incidence of liver abscess in cattle. Such action would hardly seem justifiable in view of the lack of toxicity, the absence of human health hazard and the negligible impact on the environment associated with the use of virginiamycin.

Other factors which distinguish virginiamycin from many if not all antibiotics currently approved for cattle are:

- It is a composite antibiotic and consequently less likely to induce bacterial resistance than single-entity products.
- No withdrawal period is required because it is poorly absorbed from the digestive tract of domestic animals.
- It is not, in this hemisphere, used in human therapeutics; however, it has

met the Human and Animal Health Safety Criteria for Antibiotics in Animal Feeds and is currently dispensed as a swine, turkey and broiler chicken growth enhancer.

- It is non-toxic, excreted in very low concentrations and its activity rapidly decreases.

These factors illustrate the numerous advantages virginiamycin offers over some presently available products.

In recent years, there have been significant changes in the agricultural sector of the American economy. Growing populations--both here and abroad--have increased the demand for the entire range of grain and meat food products. To meet this rising need, large scale production has become a highly technical and more efficient process. Among the numerous tools employed toward this end are a vast array of animal health products. By employing antibacterial agents to control disease and stimulate the rate of growth, a more efficient utilization of feedstuffs has been realized. The result has been an increase in the abundance of food by enriching the supply of food-animal products with the high quality protein value essential for good nutrition and health at prices within the grasp of the consumer.

Controlled clinical studies have demonstrated the potential benefits virginiamycin could offer the cattle producer in terms of increased growth rate, improved feed efficiency and reduced incidence of liver abscess resulting in lower unit production costs. In the marketplace, these benefits could be translated into increased availability of beef at a lower cost to the consuming public, in return for negligible changes in the environment.

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13. CERTIFICATION

The undersigned official certifies that the information presented is true, accurate and complete to the best of the knowledge of the firm responsible for preparation of this environmental assessment.

C. John DiCuollo — 10/26/93

C. John DiCuollo, Ph.D.* — Date
Vice President, Regulatory Affairs
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*Responsible for occupational health and environmental affairs, assuring that SmithKline Beecham Animal Health facilities worldwide and support divisions of SmithKline Beecham Corporation comply with local, state, federal and corporate standards as they apply to environmental affairs, hazardous waste disposal and worker safety.

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WATER SOLUBILITY OF VIRGINIAMYCIN (SHAKE FLASK METHOD)

Study Number: V-3008-92

Introduction

The water solubility of virginiamycin was determined at three nominal pH values; 5, 7, and 9 at 23 ± 2°C using a shake flask method described in U.S. FDA Technical Assistance Handbook (TAD 3.01). The aqueous buffer solutions were equilibrated with excess quantities of virginiamycin and the concentration of the dissolved virginiamycin was periodically determined until a plateau of concentration was reached. The concentration of virginiamycin at pH 5, 7, and 9 determined after reaching a plateau, was considered to be the solubility in water at equilibration.

Method

A mixture of ^{14}C-virginiamycin (98.1% radiochemical purity) and non-labelled virginiamycin (98.9% chemical purity) was prepared to yield a specific activity of 1190 dpm/µg (9.9113×10^2 µg/mL).

Polycarbonate tubes (15 mL capacity) with polypropylene plugs were coated with ^{14}C-virginiamycin (991 µg) by adding a standard solution to each tube and evaporating the methanol with nitrogen. The tubes were swirled periodically during drying to ensure that the test material was coated on the inner surface. The amount of virginiamycin added was in excess of the projected amount soluble in 10 mL of buffer solution, based on data obtained during a preliminary study. Ten mL of pH buffer were added to each of the tubes. The study was run in triplicate at each pH. The samples with caps were agitated on a wrist-action shaker which was placed in a dark, temperature-controlled environmental chamber at
23 ± 2°C. After high speed centrifugation (22,000 x g), triplicate aliquots (50-100 µL) were removed and analyzed for dissolved material at 3 hours, 1, 3, 11, 14, 18, 25, and 30 days after initiation. The samples were analyzed by liquid scintillation counting to determine the solubility.

Results

The water solubility of virginiamycin was determined to be 80.00 ± 6.56 µg/mL at pH 5.10, 77.08 ± 2.14 µg/mL at pH 7.26 and 54.00 ± 3.83 µg/mL at pH 9.28 at equilibrium after 18 days. Some hydrolysis of the test material was detected during the study duration by HPLC analysis of the samples. The degradation was found to be extensive at pH 9, less predominant at pH 7 and almost non-existent at pH 5.

Conclusions

Virginiamycin, with its low water solubility, is expected to be lipophilic and has a tendency to bind to soil-organic matter thereby restricting its mobility in the environment. Furthermore, its tendency to hydrolyze at pH values above 7 constitutes a possible route of degradation in the environment.

Appendix II

DETERMINATION OF THE DISSOCIATION CONSTANT OF VIRGINIAMYCIN

Study Number: V-3009-92

Introduction

A dissociation constant study with virginiamycin was conducted according to the guidelines specified in U.S. FDA Technical Assistance Handbook (TAD 3.04). A potentiometric titration was the method used.

Method

Virginiamycin with a chemical purity of 98.9% was used to conduct the study. Aqueous solutions of virginiamycin at two concentrations, one at half the solubility at pH 7 (40 ppm) and one at a higher concentration (2730 ppm), were titrated over the pH range 3-11. The titrants used were 0.001N sodium hydroxide (for pH <7) and 0.001N hydrochloric acid (for pH >7). Both titrants were standardized against an appropriate base or acid prior to use. The water used to prepare solutions was purified, deionized water from a Millipore® Milli-Q® water system and filtered through a 0.2 µm filter. Titration curves were constructed by plotting pH versus milliliter of titrant added. The first derivative curve for each titration was constructed by plotting the change in pH per milliliter of titrant added versus the milliliter of titrant added.

Results

No inflections were observed in the titration curves between pH 3 and 11 that could be distinguished from random measurement errors.

Conclusions

Virginiamycin contains no ionizable functional groups that would dissociate under environmental conditions. Therefore, pH-dependent dissociation is not a factor in the behavior of virginiamycin in the environment.

DETERMINATION OF OCTANOL/WATER PARTITION COEFFICIENT OF VIRGINIAMYCIN (SHAKE FLASK METHOD)

Study Number: V-3011-92

Introduction

The octanol/water partition coefficient of virginiamycin was determined in buffered solutions at pH 5, 7, and 9 according to the guidelines in the U.S. FDA Technical Assistance Handbook (TAD 3.02).

Method

^{14}C-virginiamycin with a radiopurity of 98.1% and a specific activity of 1190 dpm/µg was used for the study.

Solutions of ^{14}C-virginiamycin in 1-octanol (pre-saturated with water) were prepared in 48 mL Oak Ridge polycarbonate high-speed centrifuge tubes with polypropylene caps. Aqueous buffers at 5, 7, and 9 were added to the tubes, and they were placed on a rotary shaker in a temperature-controlled water bath at 25 ± 1°C. The water bath was covered with aluminum foil to prevent exposure to light. The tubes were removed from the shaker at 1, 3, and 6 hours after initiation, centrifuged (11,500 rpm), and aliquots of both layers (100 µL from 1-octanol and 875 µL from aqueous layer) were removed for liquid scintillation counting. During high-speed centrifugation, the polypropylene caps were replaced with hard plastic caps with 0-ring seals to prevent collapse of the tubes. The study was done at three dose rates, which corresponded to 4, 7.3 and 53 ppm concentrations of virginiamycin in the water layer.

Results

Equilibrium concentrations of virginiamycin in the two layers were reached after 6 hours at all three pHs in 7.3 and 53 ppm dose levels. Results are shown in Table I.

Conclusion

Since the K_{ow} values obtained are less than 100 (log K_{ow} <2) and greater than 10 (log K_{ow} >1), the test compound is not expected to significantly bioconcentrate or to sorb to organic matter in natural systems.

Table I

Octanol/Water Partition Coefficient of ^{14}C-Virginiamycin

		Concentration (mol/L)		
pH	Dose Rate (µg/mL)	Octanol Phase ($x10^{-5}$)	Aqueous Phase ($x10^{-6}$)	Average K_{ow}
5	4.0	5.257	1.023	
				51.9±1.4[1]
	7.3	9.105	1.750	
	53	63.79	12.11	52.7±1.7
7	4.0	5.291	1.125	
				54.1±5.1[2]
	7.3	9.296	1.648	
	53	66.20	12.13	54.6±2.4
9	4.0	4.730	1.383	
				32.3±1.4[3]
	7.3	8.481	2.673	
	53	58.28	19.23	30.3±1.0

[1,2,3] K_{ow} values are the averages of dose rates 4.0 and 7.3 µg/mL.

Appendix IV

ULTRAVIOLET - VISIBLE ABSORPTION SPECTRUM OF VIRGINIAMYCIN

Study Number: V-3010-92

Introduction

Ultraviolet-visible absorption spectra of virginiamycin in aqueous solutions at pH 5, 7 and 9 were obtained according to the guidelines given in U.S. FDA Technical Assistance Handbook (TAD 3.05).

Method

Aqueous buffer solutions of virginiamycin at pH 5, 7 and 9 were prepared at two different concentrations to achieve absorbances between 0.5 and 1.5. The solution concentrations were: 154 and 289 ppm at pH 5, 215 and 429 ppm at pH 7 and 123 and 270 ppm at pH 9. For concentrations below the water solubility (approximately 80 ppm) at all three pH values, the absorbance was found to be below 0.5.

The absorption spectra of the test solutions in triplicate in 1.00 cm quartz cuvettes were obtained using a spectrophotometer (Perkin-Elmer Lambda 3B UV/VIS) precalibrated using holmium oxide standard. A wavelength range of 190-800 nm was scanned at 60 nm/min at ambient temperature (ca. 23°C). The buffer solutions without virginiamycin were used as the reference at each pH during measurements.

Results

The λmax, molar absorptivity and the bandwidth are presented in Table I. There were two absorption maxima in the spectrum of the buffered solution of virginiamycin at pH 5. A single absorption maximum was observed in buffered solutions at pH 7 and 9.

Conclusion

The results of the study indicate that virginiamycin in aqueous solutions at pH 5, 7 and 9 absorb light in the ultraviolet-visible wavelength region from 290 to 800 nm. Accordingly, it is possible that virginiamycin at the surface of a water body may undergo photolytic degradation under direct sunlight.

Table I

λmax, Molar Absorptivity and Bandwidth Data for Virginiamycin Aqueous Solutions at pH 5, 7 and 9

pH	Concentrations[a] (ppm)	Mean λmax (nm)	Molar Absorptivity (L/mole*cm)	Bandwidth at A1/2 max (nm)
5	154, 289	306.1	2067	24.7
5	154, 289	353.2	1065	45.5
7	215, 429	343.3	1323	52.6
9	123, 270	338.2	3256	54.8

[a] Absorption spectra of virginiamycin were determined at two concentrations for each pH. Therefore, the λmax, molar absorptivity and bandwidth values are averages for these two concentrations.

Appendix V

SmithKline Beecham
Animal Health

MATERIAL SAFETY DATA SHEET PAGE: 1
10/01/90

VIRGINIAMYCIN
SB000115 (AHP00010)

SMITHKLINE BECKMAN ANIMAL HEALTH PRODUCTS
1600 PAOLI PIKE
WEST CHESTER, PA 19380
PHONE:1-800-877-7303

---

1. SUBSTANCE IDENTIFICATION

SUBSTANCE:
VIRGINIAMYCIN (CAS NUMBER 11006-76-1)
TRADE NAMES/SYNONYMS:
ANTIBIOTIC NUMBER 899; SK&F 7988; STAFAC; ESKALIN
CHEMICAL FAMILY:
COMPLEX ANTIBIOTIC
MOLECULAR FORMULA:
A MIXTURE OF MAINLY VIRGINIAMYCIN M1 (FACTOR M1 - C28-H35-N3-07) AND VIRGINIAMYCIN S1 (FACTOR S1 - C43-H49-N7-O10) AND SMALL QUANTITIES OF RELATED COMPONENTS.
MOLECULAR WEIGHT:
NOT APPLICABLE
SUMMARY OF HAZARDS:
PHYSICAL HAZARDS:
DUST MAY BE EXPLOSIVE.
HEALTH HAZARDS:
MAY CAUSE ALLERGIC REACTION, SKIN DERMATITIS.
MAY PRODUCE SKIN, EYE, PULMONARY IRRITATION.

---

2. COMPONENTS AND CONTAMINANTS

COMPONENT:
VIRGINIAMYCIN (100%)
OTHER CONTAMINANTS:
NONE KNOWN
EXPOSURE LIMITS:
NO PERMISSIBLE EXPOSURE LIMITS ESTABLISHED BY OSHA, ACGIH OR SMITHKLINE BECKMAN.

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3. PHYSICAL DATA

DESCRIPTION:
BROWN, GRANULATED POWDER WITH CHARACTERISTIC FERMENTATION ODOR AND VERY BITTER TASTE.
ODOR THRESHOLD:
NOT DETERMINED
DENSITY:
35-50 POUNDS/CUBIC FT
(560-801 KG/CUBIC METER)

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Animal Health

VIRGINIAMYCIN
SB000115 (AHP00010)

BOILING POINT:
NOT APPLICABLE
MELTING POINT:
NOT APPLICABLE
SPECIFIC GRAVITY:
NOT APPLICABLE
VAPOR PRESSURE:
NEGLIGIBLE
VAPOR PRESSURE:
NEGLIGIBLE
VAPOR DENSITY:
NOT APPLICABLE
% VOLATILES:
NOT APPLICABLE
EVAPORATION RATE:
NEGLIGIBLE
SOLUBILITY (SOLVENT - SOLUBILITY):
WATER - INSOLUBLE (0.6 MG/L AT PH 6)
METHANOL, ETHANOL, ACETONE, ETHYL ACETATE, CHLOROFORM, BENZENE - SOLUBLE

4. FIRE AND EXPLOSION DATA

FIRE AND EXPLOSION HAZARDS:
POWDERS CAN EXPLODE. KEEP DUST GENERATION TO A MINIMUM.
EXTINGUISHING MEDIA:
USE DRY CHEMICAL, CARBON DIOXIDE, HALON, WATER SPRAY OR STANDARD FOAM SUITABLE FOR SURROUNDING FIRE.
SPECIAL FIREFIGHTING PROCEDURES:
FIRES OF THIS MATERIAL CAN BE EXPECTED TO EMIT TOXIC FUMES. SELF-CONTAINED BREATHING APPARATUS IS RECOMMENDED FOR FIREFIGHTERS.
FLASH POINT:
NOT DETERMINED
AUTOIGNITION TEMPERATURE:
NOT DETERMINED
LOWER EXPLOSION LIMIT:
NOT DETERMINED
UPPER EXPLOSION LIMIT:
NOT DETERMINED

5. TRANSPORTATION

DEPARTMENT OF TRANSPORTATION HAZARD CLASSIFICATION AND LABELING:
NOT REGULATED (NOT CLASSIFIED AS A HAZARDOUS MATERIAL)

6. TOXICITY

LETHALITY:
VIRGINIAMYCIN PRODUCED SLIGHT TO MODERATE ORAL LETHALITY FOLLOWING A SINGLE TREATMENT IN LABORATORY ANIMALS. ORAL LD50 VALUES ARE:

MOUSE - GREATER THAN 5000 MG/KG
RAT - APPROXIMATELY 10000 MG/KG
DOG - GREATER THAN 4000 MG/KG

WHEN LETHALITY OCCURRED IN RATS AFTER A SINGLE, ORAL VIRGINIAMYCIN TREATMENT, IT WAS WITHIN 2 DAYS AFTER TREATMENT. SYMPTOMS OF POISONING INCLUDED GENERAL SIGNS OF TOXICITY SUCH AS REDUCED BODY WEIGHTS AND PROSTRATION PRIOR TO DEATH.
MUTAGENICITY:
VIRGINIAMYCIN, WAS NOT MUTAGENIC IN ONE TEST (AMES TEST) AND WEAKLY

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SmithKline Beecham
Animal Health

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VIRGINIAMYCIN
SB000115 (AHP00010)

MUTAGENIC IN ANOTHER (MOUSE LYMPHOMA TEST). NO ADVERSE EFFECTS RESULTED IN TWO TESTS FOR GENE DAMAGE (UNSCHEDULED DNA SYNTHESIS AND MOUSE MICRONUCLEUS TESTS) BUT A THIRD TEST SHOWED A WEAK EFFECT (CHINESE HAMSTER OVARY).

CARCINOGENICITY;
VIRGINIAMYCIN IS NOT LISTED AS A CARCINOGEN BY IARC, NTP OR OSHA. LIFETIME STUDIES WITH VIRGINIAMYCIN IN MICE AND RATS DEMONSTRATED NO EVIDENCE FOR CARCINOGENICITY.

REPRODUCTIVE EFFECTS:
VIRGINIAMYCIN IS NOT EXPECTED TO PRODUCE TERATOGENIC EFFECTS (BIRTH DEFECTS) OR ADVERSE EFFECTS ON MALE OR FEMALE REPRODUCTION. STUDIES WITH RATS AND MICE AND DID NOT PRODUCE SIGNIFICANT TERATOGENIC OR REPRODUCTIVE EFFECTS.

OTHER EFFECTS:
IRRITATION:
SKIN OR EYE IRRITATION HAS NOT BEEN DETERMINED.
ALLERGIC REACTIONS:
MILD TO MODERATE CONTACT DERMATITIS, PROBABLY CAUSED BY AN ALLERGIC REACTION, WAS REPORTED IN HUMANS.
CHRONIC TOXICITY:
NUMEROUS SUBCHRONIC OR CHRONIC STUDIES CONDUCTED WITH VIRGINIAMYCIN IN MICE, RATS OR DOGS FAILED TO DEMONSTRATE OBVIOUS EFFECTS THAT WOUL SUGGEST TARGET ORGANS FOR TOXICITY IN HUMANS.
PHARMACOLOGIC EFFECTS:
VIRGINIAMYCIN IS AN ANTIBIOTIC THAT IS EFFECTIVE AGAINST GRAM-POSITIVE BACTERIA.

7. HEALTH HAZARDS AND FIRST AID

PRIMARY ROUTES OF EXPOSURE:
AVOID BREATHING DUST, SKIN CONTACT, EYE CONTACT.

SKIN CONTACT:
ALLERGIC REACTIONS WITH DELAYED SYMPTOMS SUCH AS RASH, REDNESS OR SWELLING, CAN BE EXPECTED FOLLOWING IMMEDIATE OR CHRONIC SKIN CONTACT IRRITATION AFTER IMMEDIATE CONTACT CAN BE EXPECTED BUT SEVERITY OF SYMPTOMS IS NOT KNOWN.
FIRST-AID:
REMOVE CONTAMINATED CLOTHING AND WASH WITH SOAP AND WATER. IF SIGNS O IRRITATION DEVELOP, SEE A PHYSICIAN, EVEN IF SYMPTOMS ARE DELAYED.

EYE CONTACT:
EYE IRRITATION MAY OCCUR. SIGNS OF IRRITATION CAN INCLUDE REDNESS OR SWELLING OF UNKNOWN SEVERITY.
FIRST-AID:
FLUSH EYES WITH A LARGE AMOUNT OF WATER FOR AT LEAST 15 MINUTES THEN SEEK MEDICAL ATTENTION.

INHALATION:

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Animal Health

VIRGINIAMYCIN
SB000115 (AHP00010)

THE EFFECTS OF BREATHING DUST HAVE NOT BEEN DETERMINED BUT PULMONARY IRRITATION CAN BE EXPECTED. SIGNS OF TOXICITY ARE NOT KNOWN.

FIRST-AID:
REMOVE EXPOSED PERSON TO FRESH AIR. CLEAR NOSE BY BLOWING. SEE A PHYSICIAN IF SUBJECT EXPERIENCES DIFFICULTY BREATHING. IF BREATHING HAS STOPPED, PERFORM ARTIFICIAL RESPIRATION AND SEEK IMMEDIATE MEDICAL ASSISTANCE.

INGESTION:
THIS MATERIAL IS NOT EXPECTED TO PRODUCE SIGNIFICANT TOXICITY FOLLOWING INGESTION. CONSTIPATION OR DIARRHEA MAY RESULT FOLLOWING INGESTION OF LARGE QUANTITIES.

FIRST-AID:
IN THE EVENT OF OVEREXPOSURE TO THIS MATERIAL BY INGESTION, A PROPERL TRAINED PERSON SHOULD INDUCE VOMITTING BUT ONLY IF SUBJECT IS FULLY CONSCIOUS.

CONDITIONS AGGRAVATED BY EXPOSURE:
NONE KNOWN

8. REACTIVITY

CONDITIONS TO AVOID:
AVOID DUST GENERATION AND HIGH TEMPERATURES.

INCOMPATABILITY:
ONE COMPONENT, CALCIUM CARBONATE, IS NOT COMPATIBLE WITH ACIDS, ALUMINUM SALTS, AMMONIUM SALTS OR FLUORINE.

STABILITY:
STABLE AT TEMPERATURES BELOW 200 DEGREES F (93 DEGREES C).

HAZARDOUS POLYMERIZATION:
NONE KNOWN

HAZARDOUS DECOMPOSITION:
THERMAL DECOMPOSITION PRODUCTS MAY INCLUDE TOXIC AND HAZARDOUS GASES INCLUDING CALCIUM OXIDE, OXIDES OF CARBON, OXIDES OF NITROGEN, ALDEHYDES, KETONES, AND ORGANIC ACIDS.

9. STORAGE AND DISPOSAL

STORAGE:
STORE IN A COOL, DRY PLACE.

DISPOSAL:
DISPOSE OF IN AN APPROVED LANDFILL IN ACCORDANCE WITH LOCAL, STATE, AND FEDERAL REGULATIONS.

10. SPILLS AND LEAKS

FOR LIQUIDS CONTAINING THIS MATERIAL, USE ABSORBANT MATERIAL AND FLUSH AREA WITH COLD WATER, PLACE ABSORBANT MATERIAL IN AN APPROVED CONTAINER FOR

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SmithKline Beecham
Animal Health

VIRGINIAMYCIN
SB000115 (AHP00010)

DISPOSAL. SWEEP SOLID INTO AN APPROVED CONTAINER FOR DISPOSAL. KEEP DUST GENERATION TO A MINIMUM.

11. PROTECTIVE EQUIPMENT

LABORATORY:

RESPIRATORS:
A DISPOSABLE DUST RESPIRATOR SHOULD BE USED WHEN HANDLING SMALL QUANTITIES OF THIS MATERIAL OR WORK SHOULD BE PERFORMED UNDER A LABORATORY HOOD.

GLOVES:
WEAR IMPERVIOUS GLOVES.

EYE PROTECTION:
WEAR SAFETY GLASSES WITH SIDESHIELDS.

HYGIENE PRACTICES:
WASH HANDS AND ARMS THOROUGHLY AFTER HANDLING THIS MATERIAL. CLEAN UP SPILLED MATERIAL IMMEDIATELY.

VENTILATION:
USE A FUME HOOD WHEN WORKING WITH DUST.

OTHER PROTECTIVE EQUIPMENT:
WEAR LAB COAT WITH LONG SLEEVES.

AREAS WHERE LARGE QUANTITIES ARE HANDLED (PRODUCTION OR FORMULATION):

RESPIRATORS:
WEAR A DISPOSABLE DUST RESPIRATOR WHEN DUST IS PRESENT OR A HALF MASK RESPIRATOR WITH PARTICULATE CARTRIDGE.

GLOVES:
WEAR IMPERVIOUS GLOVES.

EYE PROTECTION:
WEAR GOGGLES OR A FULLFACE RESPIRATOR.

HYGIENE PRACTICES:
WASH HANDS AND ARMS THOROUGHLY AFTER HANDLING THIS MATERIAL. WEAR PROTECTIVE CLOTHING THAT IS LAUNDERED OR DISPOSED OF AFTER EACH USE. REMOVE CONTAMINATED CLOTHING. CLEAN UP SPILLS IMMEDIATELY.

VENTILATION:
USE WITH ADEQUATE MECHANICAL VENTILATION. USE LOCAL EXHAUST IN AREAS WHERE DUST IS GENERATED.

OTHER PROTECTIVE EQUIPMENT:
NONE

12. HAZARD LABEL (FOR MANUFACTURING AREAS)

VIRGINIAMYCIN - WARNING !
DUST MAY BE EXPLOSIVE - KEEP DUST TO A MINIMUM
MAY CAUSE ALLERGIC REACTION - AVOID SKIN CONTACT, BREATHING DUST
MAY CAUSE IRRITATION - AVOID SKIN CONTACT, EYE CONTACT

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Animal Health

VIRGINIAMYCIN
SB000115 (AHP00010)

CREATION DATE: 05/12/89 REVISION DATE: 05/12/89

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Animal Health

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MATERIAL SAFETY DATA SHEET PAGE: 1
10/01/90

STAFAC 500 AND 1000
SB000113 (AHP00014)

SMITHKLINE BECKMAN ANIMAL HEALTH PRODUCTS
1600 PAOLI PIKE
WEST CHESTER, PA 19380
PHONE:1-800-877-7303

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1. SUBSTANCE IDENTIFICATION

SUBSTANCES:
STAFAC (ESKALIN) 500 OR STAFAC 1000 (NO CAS NUMBER)
TRADE NAMES/SYNONYMS:
STAFAC(ESKALIN) 500 (BRAND OF VIRGINIAMYCIN) TYPE A MEDICATED ARTICLE
STAFAC 1000 (BRAND OF VIRGINIAMYCIN) TYPE A MEDICATED ARTICLE
CHEMICAL FAMILY:
ANTIBIOTIC FEED ADDITIVE
MOLECULAR FORMULA:
NOT APPLICABLE
MOLECULAR WEIGHT:
NOT APPLICABLE
SUMMARY OF HAZARDS:
PHYSICAL HAZARDS:
DUST MAY BE EXPLOSIVE.
HEALTH HAZARDS:
MAY CAUSE ALLERGIC REACTION, SKIN DERMATITIS.
MAY PRODUCE SKIN, EYE, PULMONARY IRRITATION.

---

2. COMPONENTS AND CONTAMINANTS

COMPONENT:	CAS NUMBER	PERCENT:
VIRGINIAMYCIN	11006-76-1	22-50%
CARBOXYMETHYLCELLULOSE	9004-32-4	20%
CALCIUM CARBONATE	471-34-1	29-57%
MINERAL OIL OR NORMAL LIQUID PARAFFIN	8012-95-1	1%

OTHER CONTAMINANTS:
NONE KNOWN
EXPOSURE LIMITS:
OSHA PERMISSIBLE EXPOSURE LIMIT:
NONE ESTABLISHED
ACGIH THRESHOLD LIMIT VALUE:
10 MG/CUBIC M (CALCIUM CARBONATE DUST, TIME WEIGHTED AVERAGE)
SMITHKLINE BECKMAN PERMISSIBLE IN-HOUSE LIMITS:
NONE ESTABLISHED

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3. PHYSICAL DATA

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SmithKline Beecham
Animal Health

STAFAC 500 AND 1000
SB000113 (AHP00014)

DESCRIPTION:
BROWN, GRANULATED POWDER WITH CHARACTERISTIC FERMENTATION ODOR AND VERY BITTER TASTE.

ODOR THRESHOLD:
NOT DETERMINED

DENSITY:
35-50 POUNDS/CUBIC FT
(560-801 KG/CUBIC METER)

BOILING POINT:
NOT APPLICABLE

MELTING POINT:
NOT APPLICABLE

SPECIFIC GRAVITY:
NOT APPLICABLE

VAPOR PRESSURE:
NEGLIGIBLE

VAPOR PRESSURE:
NEGLIGIBLE

VAPOR DENSITY:
NOT APPLICABLE

% VOLATILES:
NOT APPLICABLE

EVAPORATION RATE:
NEGLIGIBLE

SOLUBILITY (SOLVENT - SOLUBILITY):
WATER - INSOLUBLE (0.6 MG/L AT PH 6)

4. FIRE AND EXPLOSION DATA

FIRE AND EXPLOSION HAZARDS:
POWDERS CAN EXPLODE. KEEP DUST GENERATION TO A MINIMUM.

EXTINGUISHING MEDIA:
USE DRY CHEMICAL, CARBON DIOXIDE, HALON, WATER SPRAY OR STANDARD FOAM SUITABLE FOR SURROUNDING FIRE.

SPECIAL FIREFIGHTING PROCEDURES:
FIRES OF THIS MATERIAL CAN BE EXPECTED TO EMIT TOXIC FUMES. SELF-CONTAINED BREATHING APPARATUS IS RECOMMENDED FOR FIREFIGHTERS.

FLASH POINT:
NOT DETERMINED

AUTOIGNITION TEMPERATURE:
NOT DETERMINED

LOWER EXPLOSION LIMIT:
NOT DETERMINED

UPPER EXPLOSION LIMIT:
NOT DETERMINED

5. TRANSPORTATION

DEPARTMENT OF TRANSPORTATION HAZARD CLASSIFICATION/LABELING REQUIREMENTS:
NOT REGULATED

6. TOXICITY

THIS MIXTURE HAS NOT BEEN TESTED. EXPECTED TOXICITY IS BASED UPON INFORMATION FOR INDIVIDUAL COMPONENTS IN THE MIXTURE.

LETHALITY:
THIS MATERIAL IS EXPECTED TO PRODUCE SLIGHT TO MODERATE ORAL LETHALITY FOLLOWING A SINGLE TREATMENT IN LABORATORY ANIMALS. COMPONENTS OF THE MIXTURE, EXCEPT FOR VIRGINIAMYCIN, ARE SLIGHTLY TOXIC OR NONTOXIC. ORAL LD50 VALUES FOR VIRGINIAMYCIN ARE:

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STAFAC 500 AND 1000
SB000113 (AHP00014)

MOUSE - GREATER THAN 5000 MG/KG
RAT - APPROXIMATELY 10000 MG/KG
DOGS - GREATER THAN 4000 MG/KG

WHEN LETHALITY OCCURRED IN RATS AFTER A SINGLE, ORAL VIRGINIAMYCIN TREATMENT, IT WAS WITHIN 2 DAYS AFTER TREATMENT. SYMPTOMS OF POISONING INCLUDED GENERAL SIGNS OF TOXICITY SUCH AS REDUCED BODY WEIGHTS AND PROSTRATION PRIOR TO DEATH.

MUTAGENICITY:

THE MUTAGENICITY OF THIS MIXTURE IS NOT KNOWN. ONE COMPONENT, VIRGINIAMYCIN, WAS NOT MUTAGENIC IN ONE TEST (AMES TEST) AND WEAKLY MUTAGENIC IN ANOTHER (MOUSE LYMPHOMA TEST). NO ADVERSE EFFECTS RESULTED IN TWO TESTS FOR GENE DAMAGE (UNSCHEDULED DNA SYNTHESIS AND MOUSE MICRONUCLEUS TESTS) BUT A THIRD TEST SHOWED A WEAK EFFECT (CHINESE HAMSTER OVARY). ANOTHER COMPONENT, CARBOXYMETHYLCELLULOSE, WAS NOT MUTAGENIC IN ONE TEST (CHINESE HAMSTER OVARY).

CARCINOGENICITY:

NEITHER THIS MIXTURE NOR ANY INDIVIDUAL COMPONENT IS LISTED AS A CARCINOGEN BY IARC, NTP OR OSHA. LIFETIME STUDIES WITH VIRGINIAMYCIN IN MICE AND RATS DEMONSTRATED NO EVIDENCE FOR CARCINOGENICITY.

REPRODUCTIVE EFFECTS:

THIS MATERIAL IS NOT EXPECTED TO PRODUCE TERATOGENIC EFFECTS (BIRTH DEFECTS) OR ADVERSE EFFECTS ON MALE OR FEMALE REPRODUCTION. VIRGINIAMYCIN WAS TESTED IN RATS AND MICE AND DID NOT PRODUCE SIGNIFICANT TERATOGENIC OR REPRODUCTIVE EFFECTS. CARBOXYMETHYLCELLULOSE WAS TESTED IN MICE AND DID NOT PRODUCE EMBRYOTOXICITY OR TERATOGENICITY.

OTHER EFFECTS:

IRRITATION:

SEVERAL COMPONENTS OF THIS MIXTURE PRODUCE VARYING DEGREES OF SKIN OR EYE IRRITATION IN ANIMALS. SINCE THE MIXTURE HAS NOT BEEN TESTED, IT SHOULD BE CONSIDERED AS A POTENTIAL SKIN AND EYE IRRITANT.

ALLERGIC REACTIONS:

MILD TO MODERATE CONTACT DERMATITIS PROBABLY CAUSED BY AN ALLERGIC REACTION WAS REPORTED IN HUMANS.

CHRONIC TOXICITY:

THE CHRONIC TOXICITY OF THIS MIXTURE HAS NOT BEEN STUDIED. HOWEVER, NUMEROUS SUBCHRONIC OR CHRONIC STUDIES CONDUCTED WITH VIRGINIAMYCIN IN MICE, RATS OR DOGS FAILED TO DEMONSTRATE OBVIOUS EFFECTS THAT WOUL SUGGEST TARGET ORGANS FOR TOXICITY IN HUMANS. REMAINING COMPONENTS ARE NONTOXIC AND USED IN FOOD OR PHARMACEUTICAL AGENTS.

PHARMACOLOGIC EFFECTS:

THIS MATERIAL CONTAINS VIRGINIAMYCIN, AN ANTIBIOTIC EFFECTIVE AGAINST GRAM-POSITIVE BACTERIA.

7. HEALTH HAZARDS AND FIRST AID

PRIMARY ROUTES OF EXPOSURE:

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SmithKline Beecham
Animal Health

STAFAC 500 AND 1000
SB000113 (AHP00014)

AVOID BREATHING DUST, SKIN CONTACT, EYE CONTACT.

SKIN CONTACT:
IRRITATION OR ALLERGIC REACTIONS CAN RESULT FOLLOWING IMMEDIATE OR CHRONIC SKIN CONTACT WITH THIS MATERIAL. SYMPTOMS CAN BE IMMEDIATE OR DELAYED AND CAN INCLUDE RASH, REDNESS OR SWELLING.

FIRST-AID:
REMOVE CONTAMINATED CLOTHING AND WASH WITH SOAP AND WATER. IF SIGNS O IRRITATION DEVELOP, SEE A PHYSICIAN, EVEN IF SYMPTOMS ARE DELAYED.

EYE CONTACT:
EYE IRRITATION CAN BE EXPECTED. SIGNS OF IRRITATION CAN INCLUDE REDNESS OR SWELLING OF UNKNOWN SEVERITY.

FIRST-AID:
FLUSH EYES WITH A LARGE AMOUNT OF WATER FOR AT LEAST 15 MINUTES THEN SEEK MEDICAL ATTENTION.

INHALATION:
THE EFFECTS OF BREATHING DUST HAVE NOT BEEN DETERMINED BUT PULMONARY IRRITATION CAN BE EXPECTED. SIGNS OF TOXICITY ARE NOT KNOWN.

FIRST-AID:
REMOVE EXPOSED PERSON TO FRESH AIR. CLEAR NOSE BY BLOWING. SEE A PHYSICIAN IF SUBJECT EXPERIENCES DIFFICULTY BREATHING. IF BREATHING HAS STOPPED, PERFORM ARTIFICIAL RESPIRATION AND SEEK IMMEDIATE MEDICA ASSISTANCE.

INGESTION:
THIS MATERIAL IS NOT EXPECTED TO PRODUCE SIGNIFICANT TOXICITY FOLLOWING INGESTION. HOWEVER, INGESTION SHOULD BE AVOIDED. CONSTIPATION OR DIARRHEA MAY RESULT FOLLOWING INGESTION OF LARGE QUANTITIES.

FIRST-AID:
IN THE EVENT OF OVEREXPOSURE TO THIS MATERIAL BY INGESTION, A PROPERL TRAINED PERSON SHOULD INDUCE VOMITTING BUT ONLY IF SUBJECT IS FULLY CONSCIOUS.

CONDITIONS AGGRAVATED BY EXPOSURE:
NONE KNOWN

---

8. REACTIVITY

CONDITIONS TO AVOID:
AVOID DUST GENERATION AND HIGH TEMPERATURES.

INCOMPATABILITY:
ONE COMPONENT, CALCIUM CARBONATE, IS NOT COMPATIBLE WITH ACIDS, ALUMINUM SALTS, AMMONIUM SALTS OR FLUORINE.

STABILITY:
STABLE AT TEMPERATURES BELOW 200 DEGREES F (93 DEGREES C).

HAZARDOUS POLYMERIZATION:
NONE KNOWN

HAZARDOUS DECOMPOSITION:

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Animal Health

THERMAL DECOMPOSITION PRODUCTS MAY INCLUDE TOXIC AND HAZARDOUS GASES INCLUDING CALCIUM OXIDE, OXIDES OF CARBON, OXIDES OF NITROGEN, ALDEHYDES, KETONES, AND ORGANIC ACIDS.

9. STORAGE AND DISPOSAL

STORAGE:
STORE IN A COOL, DRY PLACE.
DISPOSAL:
DISPOSE OF IN AN APPROVED LANDFILL IN ACCORDANCE WITH LOCAL, STATE, AND FEDERAL REGULATIONS.

10. SPILLS AND LEAKS

FOR LIQUIDS CONTAINING THIS MATERIAL, USE ABSORBANT MATERIAL AND FLUSH AREA WITH COLD WATER, PLACE ABSORBANT MATERIAL IN AN APPROVED CONTAINER FOR DISPOSAL. SWEEP SOLID INTO AN APPROVED CONTAINER FOR DISPOSAL. KEEP DUST GENERATION TO A MINIMUM.

11. PROTECTIVE EQUIPMENT

LABORATORY:
RESPIRATORS:
A DISPOSABLE DUST RESPIRATOR SHOULD BE USED WHEN HANDLING SMALL QUANTITIES OF THIS MATERIAL OR WORK SHOULD BE PERFORMED UNDER A LABORATORY HOOD.
GLOVES:
WEAR IMPERVIOUS GLOVES.
EYE PROTECTION:
WEAR SAFETY GLASSES WITH SIDESHIELDS.
HYGIENE PRACTICES:
WASH HANDS AND ARMS THOROUGHLY AFTER HANDLING THIS MATERIAL. CLEAN UP SPILLED MATERIAL IMMEDIATELY.
VENTILATION:
USE A FUME HOOD WHEN WORKING WITH DUST.
OTHER PROTECTIVE EQUIPMENT:
WEAR LAB COAT WITH LONG SLEEVES.
AREAS WHERE LARGE QUANTITIES ARE HANDLED (PRODUCTION OR FORMULATION):
RESPIRATORS:
WEAR A DISPOSABLE DUST RESPIRATOR WHEN DUST IS PRESENT OR A HALF MASK RESPIRATOR WITH PARTICULATE CARTRIDGE.
GLOVES:
WEAR IMPERVIOUS GLOVES.
EYE PROTECTION:
WEAR GOGGLES OR A FULLFACE RESPIRATOR.

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SmithKline Beecham
Animal Health

STAFAC 500 AND 1000
SB000113 (AHP00014)

HYGIENE PRACTICES:
WASH HANDS AND ARMS THOROUGHLY AFTER HANDLING THIS MATERIAL. WEAR PROTECTIVE CLOTHING THAT IS LAUNDERED OR DISPOSED OF AFTER EACH USE. REMOVE CONTAMINATED CLOTHING. CLEAN UP SPILLS IMMEDIATELY.

VENTILATION:
USE WITH ADEQUATE MECHANICAL VENTILATION. USE LOCAL EXHAUST IN AREAS WHERE DUST IS GENERATED.

OTHER PROTECTIVE EQUIPMENT:
NONE

12. HAZARD LABEL (FOR MANUFACTURING AREAS)

STAFAC 500 OR STAFAC 1000
WARNING !
DUST MAY BE EXPLOSIVE - KEEP DUST TO A MINIMUM
MAY CAUSE ALLERGIC REACTION - AVOID SKIN CONTACT, BREATHING DUST
MAY CAUSE IRRITATION - AVOID SKIN CONTACT, EYE CONTACT

CREATION DATE: 05/12/89 REVISION DATE: 05/12/89

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SmithKline Beecham
Animal Health

MATERIAL SAFETY DATA SHEET PAGE: 1
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STAFAC 10,20 AND 50
SB000112 (AHP00011)

SMITHKLINE BECKMAN ANIMAL HEALTH PRODUCTS
1600 PAOLI PIKE
WEST CHESTER, PA 19380
PHONE:1-800-877-7303

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1. SUBSTANCE IDENTIFICATION

SUBSTANCES:
STAFAC 10 OR STAFAC 20 OR STAFAC 50 (NO CAS NUMBER)
TRADE NAMES/SYNONYMS:
STAFAC 10 (BRAND OF VIRGINIAMYCIN) TYPE A MEDICATED ARTICLE
STAFAC 20 (BRAND OF VIRGINIAMYCIN) TYPE A MEDICATED ARTICLE
STAFAC 50 (BRAND OF VIRGINIAMYCIN) TYPE A MEDICATED ARTICLE
CHEMICAL FAMILY:
ANTIBIOTIC FEED ADDITIVE
MOLECULAR FORMULA:
NOT APPLICABLE
MOLECULAR WEIGHT:
NOT APPLICABLE
SUMMARY OF HAZARDS:
PHYSICAL HAZARDS:
DUST MAY BE EXPLOSIVE.
SPONTANEOUS COMBUSTION MAY OCCUR.
HEALTH HAZARDS:
MAY CAUSE ALLERGIC REACTION, SKIN DERMATITIS.
MAY PRODUCE SKIN, EYE, PULMONARY IRRITATION.

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2. COMPONENTS AND CONTAMINANTS

COMPONENT:	CAS NUMBERS:	PERCENT:
VIRGINIAMYCIN	11006-76-1	2.2-11%
CARBOXYMETHYLCELLULOSE	9004-32-4	0.88-4.4%
CALCIUM CARBONATE	471-34-1	2.4-10.8%
MINERAL OIL OR NORMAL LIQUID PARAFFIN	8012-95-1	0.044-0.2%
RICE HULLS AND/OR DRIED DISTILLERS GRAINS WITH SOLUBLES	NONE	89-97%

OTHER CONTAMINANTS:
NONE KNOWN
EXPOSURE LIMITS:
OSHA PERMISSIBLE EXPOSURE LIMIT:
NONE ESTABLISHED
ACGIH THRESHOLD LIMIT VALUE:
10 MG/CUBIC METER (CALCIUM CARBONATE DUST, TIME WEIGHTED AVERAGE)
SMITHKLINE BECKMAN PERMISSIBLE IN-HOUSE LIMITS:

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Animal Health

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STAFAC 10,20 AND 50
SB000112 (AHP00011)

NONE ESTABLISHED

3. PHYSICAL DATA

DESCRIPTION:
BROWN, GRANULATED POWDER WITH CHARACTERISTIC FERMENTATION ODOR AND VERY BITTER TASTE.

ODOR THRESHOLD:
NOT DETERMINED

DENSITY:
35-50 POUNDS/CUBIC FT
(560-801 KG/CUBIC METER)

BOILING POINT:
NOT APPLICABLE

MELTING POINT:
NOT APPLICABLE

SPECIFIC GRAVITY:
NOT APPLICABLE

VAPOR PRESSURE:
NEGLIGIBLE

VAPOR PRESSURE:
NEGLIGIBLE

VAPOR DENSITY:
NOT APPLICABLE

% VOLATILES:
NOT APPLICABLE

EVAPORATION RATE:
NEGLIGIBLE

SOLUBILITY (SOLVENT - SOLUBILITY):
WATER - INSOLUBLE (0.6 MG/L AT PH 6)

4. FIRE AND EXPLOSION DATA

FIRE AND EXPLOSION HAZARDS:
POWDERS CAN EXPLODE. KEEP DUST GENERATION TO A MINIMUM.

EXTINGUISHING MEDIA:
USE DRY CHEMICAL, CARBON DIOXIDE, HALON, WATER SPRAY OR STANDARD FOAM SUITABLE FOR SURROUNDING FIRE.

SPECIAL FIREFIGHTING PROCEDURES:
FIRES OF THIS MATERIAL CAN BE EXPECTED TO EMIT TOXIC FUMES. SELF-CONTAINED BREATHING APPARATUS IS RECOMMENDED FOR FIREFIGHTERS.

FLASH POINT:
NOT DETERMINED

AUTOIGNITION TEMPERATURE:
NOT DETERMINED

LOWER EXPLOSION LIMIT:
NOT DETERMINED

UPPER EXPLOSION LIMIT:
NOT DETERMINED

5. TRANSPORTATION

DEPARTMENT OF TRANSPORTATION HAZARD CLASSIFICATION/LABELING REQUIREMENTS:
NOT REGULATED

6. TOXICITY

THIS MIXTURE HAS NOT BEEN TESTED. EXPECTED TOXICITY IS BASED UPON INFORMATION FOR INDIVIDUAL COMPONENTS IN THE MIXTURE.

LETHALITY:

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Animal Health

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STAFAC 10,20 AND 50
SB000112 (AHP00011)

THIS MATERIAL IS EXPECTED TO PRODUCE SLIGHT TO MODERATE ORAL LETHALITY FOLLOWING A SINGLE TREATMENT IN LABORATORY ANIMALS. COMPONENTS OF THE MIXTURE, EXCEPT FOR VIRGINIAMYCIN, ARE SLIGHTLY TOXIC OR NONTOXIC. ORAL LD50 VALUES FOR VIRGINIAMYCIN ARE:

MOUSE - GREATER THAN 5000 MG/KG
RAT - APPROXIMATELY 10000 MG/KG
DOGS - GREATER THAN 4000 MG/KG

WHEN LETHALITY OCCURRED IN RATS AFTER A SINGLE, ORAL VIRGINIAMYCIN TREATMENT, IT WAS WITHIN 2 DAYS AFTER TREATMENT. SYMPTOMS OF POISONING INCLUDED GENERAL SIGNS OF TOXICITY SUCH AS REDUCED BODY WEIGHTS AND PROSTRATION PRIOR TO DEATH.

MUTAGENICITY:

THE MUTAGENICITY OF THIS MIXTURE IS NOT KNOWN. ONE COMPONENT, VIRGINIAMYCIN, WAS NOT MUTAGENIC IN ONE TEST (AMES TEST) AND WEAKLY MUTAGENIC IN ANOTHER (MOUSE LYMPHOMA TEST). NO ADVERSE EFFECTS RESULTED IN TWO TESTS FOR GENE DAMAGE (UNSCHEDULED DNA SYNTHESIS AND MOUSE MICRONUCLEUS TESTS) BUT A THIRD TEST SHOWED A WEAK EFFECT (CHINESE HAMSTER OVARY). ANOTHER COMPONENT, CARBOXYMETHYLCELLULOSE, WAS NOT MUTAGENIC IN ONE TEST (CHINESE HAMSTER OVARY).

CARCINOGENICITY:

NEITHER THIS MIXTURE NOR ANY INDIVIDUAL COMPONENT IS LISTED AS A CARCINOGEN BY IARC, NTP OR OSHA. LIFETIME STUDIES WITH VIRGINIAMYCIN IN MICE AND RATS DEMONSTRATED NO EVIDENCE FOR CARCINOGENICITY.

REPRODUCTIVE EFFECTS:

THIS MATERIAL IS NOT EXPECTED TO PRODUCE TERATOGENIC EFFECTS (BIRTH DEFECTS) OR ADVERSE EFFECTS ON MALE OR FEMALE REPRODUCTION. VIRGINIAMYCIN WAS TESTED IN RATS AND MICE AND DID NOT PRODUCE SIGNIFICANT TERATOGENIC OR REPRODUCTIVE EFFECTS. CARBOXYMETHYLCELLULOSE WAS TESTED IN MICE AND DID NOT PRODUCE EMBRYOTOXICITY OR TERATOGENICITY.

OTHER EFFECTS:

IRRITATION:

SEVERAL COMPONENTS OF THIS MIXTURE PRODUCE VARYING DEGREES OF SKIN OR EYE IRRITATION IN ANIMALS. SINCE THE MIXTURE HAS NOT BEEN TESTED, IT SHOULD BE CONSIDERED AS A POTENTIAL SKIN AND EYE IRRITANT.

ALLERGIC REACTIONS:

MILD TO MODERATE CONTACT DERMATITIS PROBABLY CAUSED BY AN ALLERGIC REACTION WAS REPORTED IN HUMANS.

CHRONIC TOXICITY:

THE CHRONIC TOXICITY OF THIS MIXTURE HAS NOT BEEN STUDIED. HOWEVER, NUMEROUS SUBCHRONIC OR CHRONIC STUDIES CONDUCTED WITH VIRGINIAMYCIN IN MICE, RATS OR DOGS FAILED TO DEMONSTRATE OBVIOUS EFFECTS THAT WOUL SUGGEST TARGET ORGANS FOR TOXICITY IN HUMANS. REMAINING COMPONENTS ARE NONTOXIC AND USED IN FOOD OR PHARMACEUTICAL AGENTS.

PHARMACOLOGIC EFFECTS:

THIS MATERIAL CONTAINS VIRGINIAMYCIN, AN ANTIBIOTIC EFFECTIVE AGAINST GRAM-POSITIVE BACTERIA.

SMITHKLINE BEECHAM CORPORATION

SmithKline Beecham
Animal Health

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STAFAC 10,20 AND 50
SB000112 (AHP00011)

7. HEALTH HAZARDS AND FIRST AID

PRIMARY ROUTES OF EXPOSURE:
AVOID BREATHING DUST, SKIN CONTACT, EYE CONTACT.

SKIN CONTACT:
IRRITATION OR ALLERGIC REACTIONS CAN RESULT FOLLOWING IMMEDIATE OR CHRONIC SKIN CONTACT WITH THIS MATERIAL. SYMPTOMS CAN BE IMMEDIATE OR DELAYED AND CAN INCLUDE RASH, REDNESS OR SWELLING.

FIRST-AID:
REMOVE CONTAMINATED CLOTHING AND WASH WITH SOAP AND WATER. IF SIGNS O IRRITATION DEVELOP, SEE A PHYSICIAN, EVEN IF SYMPTOMS ARE DELAYED.

EYE CONTACT:
EYE IRRITATION CAN BE EXPECTED. SIGNS OF IRRITATION CAN INCLUDE REDNESS OR SWELLING OF UNKNOWN SEVERITY.

FIRST-AID:
FLUSH EYES WITH A LARGE AMOUNT OF WATER FOR AT LEAST 15 MINUTES THEN SEEK MEDICAL ATTENTION.

INHALATION:
THE EFFECTS OF BREATHING DUST HAVE NOT BEEN DETERMINED BUT PULMONARY IRRITATION CAN BE EXPECTED. SIGNS OF TOXICITY ARE NOT KNOWN.

FIRST-AID:
REMOVE EXPOSED PERSON TO FRESH AIR. CLEAR NOSE BY BLOWING. SEE A PHYSICIAN IF SUBJECT EXPERIENCES DIFFICULTY BREATHING. IF BREATHING HAS STOPPED, PERFORM ARTIFICIAL RESPIRATION AND SEEK IMMEDIATE MEDICA ASSISTANCE.

INGESTION:
THIS MATERIAL IS NOT EXPECTED TO PRODUCE SIGNIFICANT TOXICITY FOLLOWING INGESTION. HOWEVER, INGESTION SHOULD BE AVOIDED. CONSTIPATION OR DIARRHEA MAY RESULT FOLLOWING INGESTION OF LARGE QUANTITIES.

FIRST-AID:
IN THE EVENT OF OVEREXPOSURE TO THIS MATERIAL BY INGESTION, A PROPERL TRAINED PERSON SHOULD INDUCE VOMITTING BUT ONLY IF SUBJECT IS FULLY CONSCIOUS.

CONDITIONS AGGRAVATED BY EXPOSURE:
NONE KNOWN

8. REACTIVITY

CONDITIONS TO AVOID:
AVOID DUST GENERATION AND HIGH TEMPERATURES. KEEP DRY.

INCOMPATABILITY:
ONE COMPONENT, CALCIUM CARBONATE, IS NOT COMPATIBLE WITH ACIDS, ALUMINUM SALTS, AMMONIUM SALTS OR FLUORINE.

STABILITY:

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Animal Health

STAFAC 10,20 AND 50
SB000112 (AHP00011)

STABLE AT TEMPERATURES BELOW 200 DEGREES F (93 DEGREES C).
HAZARDOUS POLYMERIZATION:
NONE KNOWN
HAZARDOUS DECOMPOSITION:
THERMAL DECOMPOSITION PRODUCTS MAY INCLUDE TOXIC AND HAZARDOUS GASES INCLUDING CALCIUM OXIDE, OXIDES OF CARBON, OXIDES OF NITROGEN, ALDEHYDES, KETONES, AND ORGANIC ACIDS.

9. STORAGE AND DISPOSAL

STORAGE:
STORE IN A COOL, DRY PLACE.
DISPOSAL:
DISPOSE OF IN AN APPROVED LANDFILL IN ACCORDANCE WITH LOCAL, STATE, AND FEDERAL REGULATIONS.

10. SPILLS AND LEAKS

FOR LIQUIDS CONTAINING THIS MATERIAL USE ABSORBANT MATERIAL AND FLUSH AREA WITH COLD WATER, PLACE ABSORBANT MATERIAL IN AN APPROVED CONTAINER FOR DISPOSAL. SWEEP SOLID INTO AN APPROVED CONTAINER FOR DISPOSAL. KEEP DUST GENERATION TO A MINIMUM.

11. PROTECTIVE EQUIPMENT

LABORATORY:
RESPIRATORS:
A DISPOSABLE DUST RESPIRATOR SHOULD BE USED WHEN HANDLING SMALL QUANTITIES OF THIS MATERIAL OR WORK SHOULD BE PERFORMED UNDER A LABORATORY HOOD.
GLOVES:
WEAR IMPERVIOUS GLOVES.
EYE PROTECTION:
WEAR SAFETY GLASSES WITH SIDESHIELDS.
HYGIENE PRACTICES:
WASH HANDS AND ARMS THOROUGHLY AFTER HANDLING THIS MATERIAL. CLEAN UP SPILLED MATERIAL IMMEDIATELY.
VENTILATION:
USE A FUME HOOD WHEN WORKING WITH DUST.
OTHER PROTECTIVE EQUIPMENT:
WEAR LAB COAT WITH LONG SLEEVES.
AREAS WHERE LARGE QUANTITIES ARE HANDLED (PRODUCTION OR FORMULATION):
RESPIRATORS:
WEAR A DISPOSABLE DUST RESPIRATOR WHEN DUST IS PRESENT OR A HALF MASK RESPIRATOR WITH PARTICULATE CARTRIDGE.
GLOVES:

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WEAR IMPERVIOUS GLOVES.

EYE PROTECTION:
WEAR GOGGLES OR A FULLFACE RESPIRATOR.

HYGIENE PRACTICES:
WASH HANDS AND ARMS THOROUGHLY AFTER HANDLING THIS MATERIAL. WEAR PROTECTIVE CLOTHING THAT IS LAUNDERED OR DISPOSED OF AFTER EACH USE. REMOVE CONTAMINATED CLOTHING. CLEAN UP SPILLS IMMEDIATELY.

VENTILATION:
USE WITH ADEQUATE MECHANICAL VENTILATION. USE LOCAL EXHAUST IN AREAS WHERE DUST IS GENERATED.

OTHER PROTECTIVE EQUIPMENT:
NONE

12. HAZARD LABEL (FOR MANUFACTURING AREAS)

STAFAC 10 OR STAFAC 20 OR STAFAC 100
WARNING !
DUST MAY BE EXPLOSIVE - KEEP DUST TO A MINIMUM
MAY CAUSE ALLERGIC REACTION - AVOID SKIN CONTACT, BREATHING DUST
MAY CAUSE IRRITATION - AVOID SKIN CONTACT, EYE CONTACT
SPONTANEOUS COMBUSTION MAY OCCUR - KEEP DRY

CREATION DATE: 05/12/89 REVISION DATE: 05/12/89

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SmithKline Beecham
Animal Health

MATERIAL SAFETY DATA SHEET PAGE: 1
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ESKALIN/STAFAC 20 OR ESKALIN/STAFAC 100
SB000114 (AHP00015)

SMITHKLINE BECKMAN ANIMAL HEALTH PRODUCTS
1600 PAOLI PIKE
WEST CHESTER, PA 19380
PHONE:1-800-877-7303

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1. SUBSTANCE IDENTIFICATION

SUBSTANCES:
ESKALIN/STAFAC 20 OR ESKALIN/STAFAC 100 (NO CAS NUMBER)
TRADE NAMES/SYNONYMS:
ESKALIN/STAFAC 20 (BRAND OF VIRGINIAMYCIN) PREMIX
ESKALIN/STAFAC 100 (BRAND OF VIRGINIAMYCIN) PREMIX
CHEMICAL FAMILY:
ANTIBIOTIC FEED ADDITIVE
MOLECULAR FORMULA:
NOT APPLICABLE
MOLECULAR WEIGHT:
NOT APPLICABLE
SUMMARY OF HAZARDS:
PHYSICAL HAZARDS:
DUST MAY BE EXPLOSIVE.
HEALTH HAZARDS:
MAY CAUSE ALLERGIC REACTION, SKIN DERMATITIS.
MAY PRODUCE SKIN, EYE, PULMONARY IRRITATION.

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2. COMPONENTS AND CONTAMINANTS

COMPONENT:	CAS NUMBERS:	PERCENT:
VIRGINIAMYCIN	11006-76-1	2.0-10%
CARBOXYMETHYLCELLULOSE	9004-32-4	0.8-4%
CALCIUM CARBONATE	471-34-1	96.8-85.8%
MINERAL OIL OR NORMAL LIQUID PARAFFIN	8012-95-1	0.04-0.2%

OTHER CONTAMINANTS:
NONE KNOWN
EXPOSURE LIMITS:
OSHA PERMISSIBLE EXPOSURE LIMIT:
NONE ESTABLISHED
ACGIH THRESHOLD LIMIT VALUE:
10 MG/CUBIC M (CALCIUM CARBONATE DUST, TIME WEIGHTED AVERAGE)
SMITHKLINE BECKMAN PERMISSIBLE IN-HOUSE LIMITS:
NONE ESTABLISHED

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3. PHYSICAL DATA

1600 Paoli Pike, PO Box 2650, West Chester, PA 19380-6014 Telephone (215) 251 [illegible]

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SmithKline Beecham
Animal Health

ESKALIN/STAFAC 20 OR ESKALIN/STAFAC 100
SB000114 (AHP00015)

DESCRIPTION:
BROWN, GRANULATED POWDER WITH CHARACTERISTIC FERMENTATION ODOR AND VERY BITTER TASTE.

ODOR THRESHOLD:
NOT DETERMINED

DENSITY:
35-50 POUNDS/CUBIC FT
(560-801 KG/CUBIC METER)

BOILING POINT:
NOT APPLICABLE

MELTING POINT:
NOT APPLICABLE

SPECIFIC GRAVITY:
NOT APPLICABLE

VAPOR PRESSURE:
NEGLIGIBLE

VAPOR PRESSURE:
NEGLIGIBLE

VAPOR DENSITY:
NOT APPLICABLE

% VOLATILES:
NOT APPLICABLE

EVAPORATION RATE:
NEGLIGIBLE

SOLUBILITY (SOLVENT - SOLUBILITY):
WATER - INSOLUBLE (0.6 MG/L AT PH 6)

4. FIRE AND EXPLOSION DATA

FIRE AND EXPLOSION HAZARDS:
POWDER CAN EXPLODE. KEEP DUST GENERATION TO A MINIMUM.

EXTINGUISHING MEDIA:
USE DRY CHEMICAL, CARBON DIOXIDE, HALON, WATER SPRAY OR STANDARD FOAM SUITABLE FOR SURROUNDING FIRE.

SPECIAL FIREFIGHTING PROCEDURES:
FIRES OF THIS MATERIAL CAN BE EXPECTED TO EMIT TOXIC FUMES. SELF-CONTAINED BREATHING APPARATUS IS RECOMMENDED FOR FIREFIGHTERS.

FLASH POINT:
NOT DETERMINED

AUTOIGNITION TEMPERATURE:
NOT DETERMINED

LOWER EXPLOSION LIMIT:
NOT DETERMINED

UPPER EXPLOSION LIMIT:
NOT DETERMINED

5. TRANSPORTATION

DEPARTMENT OF TRANSPORTATION HAZARD CLASSIFICATION/LABELING REQUIREMENTS:
NOT REGULATED

6. TOXICITY

THIS MIXTURE HAS NOT BEEN TESTED. EXPECTED TOXICITY IS BASED UPON INFORMATION FOR INDIVIDUAL COMPONENTS IN THE MIXTURE.

LETHALITY:
THIS MATERIAL IS EXPECTED TO PRODUCE SLIGHT TO MODERATE ORAL LETHALITY FOLLOWING A SINGLE TREATMENT IN LABORATORY ANIMALS. COMPONENTS OF THE MIXTURE, EXCEPT FOR VIRGINIAMYCIN, ARE SLIGHTLY TOXIC OR NONTOXIC. ORAL LD50 VALUES FOR VIRGINIAMYCIN ARE:

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MOUSE - GREATER THAN 5000 MG/KG
RAT - APPROXIMATELY 10000 MG/KG
DOGS - GREATER THAN 4000 MG/KG

WHEN LETHALITY OCCURRED IN RATS AFTER A SINGLE, ORAL VIRGINIAMYCIN TREATMENT, IT WAS WITHIN 2 DAYS AFTER TREATMENT. SYMPTOMS OF POISONING INCLUDED GENERAL SIGNS OF TOXICITY SUCH AS REDUCED BODY WEIGHTS AND PROSTRATION PRIOR TO DEATH.

MUTAGENICITY:

THE MUTAGENICITY OF THIS MIXTURE IS NOT KNOWN. ONE COMPONENT, VIRGINIAMYCIN, WAS NOT MUTAGENIC IN ONE TEST (AMES TEST) AND WEAKLY MUTAGENIC IN ANOTHER (MOUSE LYMPHOMA TEST). NO ADVERSE EFFECTS RESULTED IN TWO TESTS FOR GENE DAMAGE (UNSCHEDULED DNA SYNTHESIS AND MOUSE MICRONUCLEUS TESTS) BUT A THIRD TEST SHOWED A WEAK EFFECT (CHINESE HAMSTER OVARY). ANOTHER COMPONENT, CARBOXYMETHYLCELLULOSE, WAS NOT MUTAGENIC IN ONE TEST (CHINESE HAMSTER OVARY).

CARCINOGENICITY:

NEITHER THIS MIXTURE NOR ANY INDIVIDUAL COMPONENT IS LISTED AS A CARCINOGEN BY IARC, NTP OR OSHA. LIFETIME STUDIES WITH VIRGINIAMYCIN IN MICE AND RATS DEMONSTRATED NO EVIDENCE FOR CARCINOGENICITY.

REPRODUCTIVE EFFECTS:

THIS MATERIAL IS NOT EXPECTED TO PRODUCE TERATOGENIC EFFECTS (BIRTH DEFECTS) OR ADVERSE EFFECTS ON MALE OR FEMALE REPRODUCTION. VIRGINIAMYCIN WAS TESTED IN RATS AND MICE AND DID NOT PRODUCE SIGNIFICANT TERATOGENIC OR REPRODUCTIVE EFFECTS. CARBOXYMETHYLCELLULOSE WAS TESTED IN MICE AND DID NOT PRODUCE EMBRYOTOXICITY OR TERATOGENICITY.

OTHER EFFECTS:

IRRITATION:

SEVERAL COMPONENTS OF THIS MIXTURE PRODUCE VARYING DEGREES OF SKIN OR EYE IRRITATION IN ANIMALS. SINCE THE MIXTURE HAS NOT BEEN TESTED, IT SHOULD BE CONSIDERED AS A POTENTIAL SKIN AND EYE IRRITANT.

ALLERGIC REACTIONS:

MILD TO MODERATE CONTACT DERMATITIS PROBABLY CAUSED BY AN ALLERGIC REACTION WAS REPORTED IN HUMANS.

CHRONIC TOXICITY:

THE CHRONIC TOXICITY OF THIS MIXTURE HAS NOT BEEN STUDIED. HOWEVER, NUMEROUS SUBCHRONIC OR CHRONIC STUDIES CONDUCTED WITH VIRGINIAMYCIN IN MICE, RATS OR DOGS FAILED TO DEMONSTRATE OBVIOUS EFFECTS THAT WOUL SUGGEST TARGET ORGANS FOR TOXICITY IN HUMANS. REMAINING COMPONENTS ARE NONTOXIC AND USED IN FOOD OR PHARMACEUTICAL AGENTS.

PHARMACOLOGIC EFFECTS:

THIS MATERIAL CONTAINS VIRGINIAMYCIN, AN ANTIBIOTIC EFFECTIVE AGAINST GRAM-POSITIVE BACTERIA.

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7. HEALTH HAZARDS AND FIRST AID

PRIMARY ROUTES OF EXPOSURE:

1600 Paoli Pike PO Box 7440 [illegible] PA [illegible] Telephone (215) 251 7400 Fax (215) 251 7590

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Animal Health

AVOID BREATHING DUST, SKIN CONTACT, EYE CONTACT.

SKIN CONTACT:
IRRITATION OR ALLERGIC REACTIONS CAN RESULT FOLLOWING IMMEDIATE OR CHRONIC SKIN CONTACT WITH THIS MATERIAL. SYMPTOMS CAN BE IMMEDIATE OR DELAYED AND CAN INCLUDE RASH, REDNESS OR SWELLING.

FIRST-AID:
REMOVE CONTAMINATED CLOTHING AND WASH WITH SOAP AND WATER. IF SIGNS O IRRITATION DEVELOP, SEE A PHYSICIAN, EVEN IF SYMPTOMS ARE DELAYED.

EYE CONTACT:
EYE IRRITATION CAN BE EXPECTED. SIGNS OF IRRITATION CAN INCLUDE REDNESS OR SWELLING OF UNKNOWN SEVERITY.

FIRST-AID:
FLUSH EYES WITH A LARGE AMOUNT OF WATER FOR AT LEAST 15 MINUTES THEN SEEK MEDICAL ATTENTION.

INHALATION:
THE EFFECTS OF BREATHING DUST HAVE NOT BEEN DETERMINED BUT PULMONARY IRRITATION CAN BE EXPECTED. SIGNS OF TOXICITY ARE NOT KNOWN.

FIRST-AID:
REMOVE EXPOSED PERSON TO FRESH AIR. CLEAR NOSE BY BLOWING. SEE A PHYSICIAN IF SUBJECT EXPERIENCES DIFFICULTY BREATHING. IF BREATHING HAS STOPPED, PERFORM ARTIFICIAL RESPIRATION AND SEEK IMMEDIATE MEDICA ASSISTANCE.

INGESTION:
THIS MATERIAL IS NOT EXPECTED TO PRODUCE SIGNIFICANT TOXICITY FOLLOWING INGESTION. HOWEVER, INGESTION SHOULD BE AVOIDED. CONSTIPATION OR DIARRHEA MAY RESULT FOLLOWING INGESTION OF LARGE QUANTITIES.

FIRST-AID:
IN THE EVENT OF OVEREXPOSURE TO THIS MATERIAL BY INGESTION, A PROPERL TRAINED PERSON SHOULD INDUCE VOMITTING BUT ONLY IF SUBJECT IS FULLY CONSCIOUS.

CONDITIONS AGGRAVATED BY EXPOSURE:
NONE KNOWN

8. REACTIVITY

CONDITIONS TO AVOID:
AVOID DUST GENERATION AND HIGH TEMPERATURES.

INCOMPATABILITY:
ONE COMPONENT, CALCIUM CARBONATE, IS NOT COMPATIBLE WITH ACIDS, ALUMINUM SALTS, AMMONIUM SALTS OR FLUORINE.

STABILITY:
STABLE AT TEMPERATURES BELOW 200 DEGREES F (93 DEGREES C).

HAZARDOUS POLYMERIZATION:
NONE KNOWN

HAZARDOUS DECOMPOSITION:

1250 Pond Rd., P.O. Box 2950, West Chester, PA 19380-6014 Telephone (215) 251 7400 Fax (215) 251 7580.

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THERMAL DECOMPOSITION PRODUCTS MAY INCLUDE TOXIC AND HAZARDOUS GASES INCLUDING CALCIUM OXIDE, OXIDES OF CARBON, OXIDES OF NITROGEN, ALDEHYDES, KETONES, AND ORGANIC ACIDS.

9. STORAGE AND DISPOSAL

STORAGE:
STORE IN A COOL, DRY PLACE.
DISPOSAL:
DISPOSE OF IN AN APPROVED LANDFILL IN ACCORDANCE WITH LOCAL, STATE, AND FEDERAL REGULATIONS.

10. SPILLS AND LEAKS

FOR LIQUIDS CONTAINING THIS MATERIAL, USE ABSORBANT MATERIAL AND FLUSH AREA WITH COLD WATER, PLACE ABSORBANT MATERIAL IN AN APPROVED CONTAINER FOR DISPOSAL. SWEEP SOLID INTO AN APPROVED CONTAINER FOR DISPOSAL. KEEP DUST GENERATION TO A MINIMUM.

11. PROTECTIVE EQUIPMENT

LABORATORY:
RESPIRATORS:
A DISPOSABLE DUST RESPIRATOR SHOULD BE USED WHEN HANDLING SMALL QUANTITIES OF THIS MATERIAL OR WORK SHOULD BE PERFORMED UNDER A LABORATORY HOOD.
GLOVES:
WEAR IMPERVIOUS GLOVES.
EYE PROTECTION:
WEAR SAFETY GLASSES WITH SIDESHIELDS.
HYGIENE PRACTICES:
WASH HANDS AND ARMS THOROUGHLY AFTER HANDLING THIS MATERIAL. CLEAN UP SPILLED MATERIAL IMMEDIATELY.
VENTILATION:
USE A FUME HOOD WHEN WORKING WITH DUST.
OTHER PROTECTIVE EQUIPMENT:
WEAR LAB COAT WITH LONG SLEEVES.
AREAS WHERE LARGE QUANTITIES ARE HANDLED (PRODUCTION OR FORMULATION):
RESPIRATORS:
WEAR A DISPOSABLE DUST RESPIRATOR WHEN DUST IS PRESENT OR A HALF MASK RESPIRATOR WITH PARTICULATE CARTRIDGE.
GLOVES:
WEAR IMPERVIOUS GLOVES.
EYE PROTECTION:
WEAR GOGGLES OR A FULLFACE RESPIRATOR.
HYGIENE PRACTICES:

1600 Paoli Pike, PO Box 2650, West Chester, PA 19380-6014. Telephone (215) 251 7400 Fax (215) 251 7580

SmithKline Beecham
Animal Health

WASH HANDS AND ARMS THOROUGHLY AFTER HANDLING THIS MATERIAL. WEAR PROTECTIVE CLOTHING THAT IS LAUNDERED OR DISPOSED OF AFTER EACH USE. REMOVE CONTAMINATED CLOTHING. CLEAN UP SPILLS IMMEDIATELY.

VENTILATION:
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OTHER PROTECTIVE EQUIPMENT:
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VIRGINIAMYCIN METABOLISM IN CATTLE RUMEN FLUID

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ABSTRACT:

The antibiotic virginiamycin (VM) was extensively metabolized in cattle rumen fluid *in vitro*. The antimicrobial activity of VM decreased rapidly with an initial half-life of approximately 8 hr. In contrast, in buffer at near neutral pH, VM maintained its full activity for at least 24 hr. Four metabolites were isolated and identified using a combination of spectral techniques including FAB MS-MS, LC-MS, and 500 MHz NMR. The metabolites were all derived from reduction of the major component of virginiamycin, factor M. The minor component, factor S, was not metabolized in cattle rumen fluid. The metabolic pathways involved included C — C and C — O reduction as well as dehydroxylation. All metabolites were found to have less antimicrobial activity than the parent factor M. In addition to the metabolites, two factor M decomposition products were isolated after incubation of VM in buffer alone. These two products resulted from the dehydration of factor M and were shown to interconvert at room temperature.

Virginiamycin is an antibiotic active against gram-positive bacteria in the gut (1). The drug is a fermentation product of *Streptomyces virginiae* and is composed of two major factors (M_1 and S_1), which function synergistically (2) when combined in an optimum ratio of 4:1 (M:S). The structures of these two factors are shown in fig. 1. The compound has long been known to produce favorable responses in growth and/or feed efficiency in broilers (3–5), turkey poults (6), and swine (7) when used as a feed additive (Stafac) in the diet. Because similar growth-promotant effects have been found in cattle (8), metabolism data were required to support product clearance through various worldwide regulatory agencies for this species. Previous studies concerning the *in vivo* metabolism of virginiamycin have been conducted in turkeys, cattle, and rats (9, 10); however, the tissue residue profile was such that no metabolite identification was possible. In the present study, we report the isolation and identification of four metabolites of virginiamycin factor M after incubation in cattle rumen fluid *in vitro*.

Materials and Methods

Animals. Mature (1–2 years old, 1000 lb), Hereford cross, rumen-fistulated steers were used exclusively in the study. The animals were maintained on diet AN (Fisher and Son, Co., Malvern, PA.) and received approximately 24 lb of feed per day.

Test Substance. [^{14}C]Virginiamycin was extracted from fermentation broths of *Streptomyces virginiae*. When prepared in this manner using [^{14}C]acetate, [^{14}C]lysine, and [^{14}C]phenylalanine as precursors, the isolated product becomes relatively uniformly labeled, in that all the carbon atoms have some degree of incorporation but the extent varies with each position. Factors M and S were purified separately by HPLC and then recombined in a 4:1 (w/w) ratio. Appropriate amounts of nonradiolabeled reference standards were added to adjust each factor to identical specific activities (0.2–0.8 mCi/g). The final [^{14}C]virginiamycin assayed to a chemical and radio-purity of >98% (TLC, HPLC) with a microbiological potency (9) of at least 193% activity (pad plate diffusion assay using *Corynebacterium xerosis*).

Rumen Fluid Collection. Two hr after the morning feeding (approximately 12 lb) the fistula stopper was removed (nonaseptic) and the rumen contents were mixed by hand *in situ*. The rumen contents were scooped into a funnel lined with two layers of cheesecloth and compressed so that the fluid was filtered and collected in a 4-liter plastic bottle. The collected fluid was refiltered through an additional two layers of cheesecloth and mixed 1:1 (v/v) with McDougall's (11) nutrient solution (pH 6.5) in a vessel maintained under an atmosphere of carbon dioxide, with constant stirring.

Fermentation. Before the addition of rumen fluid, 100 mg of corn starch and 400 mg of Solkafloc BW-40 (cellulose) were weighed into 50-ml Erlenmeyer flasks to serve as a food source for the rumen bacteria. Virginiamycin was dissolved in ethanol (2.46 mg/ml) and an appropriate amount was pipetted directly onto the starch/cellulose. The ethanol was allowed to evaporate for approximately 5 min and 20 ml of rumen fluid were added to each flask. The flasks were swirled, gassed with CO_2 for 10–15 sec, and fitted with a stopper containing a one-way ball valve for releasing internal pressure. The incubation was conducted in a Precision model 50 shaking water bath (100 oscillations/min) at a temperature of 39°C for up to 48 hr.

For metabolite isolation studies, the incubation procedure was scaled up to 500-ml Erlenmeyer flasks containing 200 ml of rumen fluid and 3.69 mg of [^{14}C]virginiamycin.

VFA[1] Analysis. Fermentation was stopped by the addition of 0.2 ml of 25% metaphosphoric acid per ml of rumen fluid. The flask was capped, swirled, (frozen if not analyzed immediately), and centrifuged at 4°C, 50,000*g* for 20 min. GC analyses of the supernatant for acetic, propionic, isobutyric, butyric, valeric, and isovaleric acids were determined from an external standard curve using a HP 5840A gas chromatograph with an HP7672A autosampler and an HP5840A integrator interfaced with a Digital Minc RX02 computer. A 6-ft stainless steel column (1/8 inch i.d.; Supelco 10% SP1200/1% H_3PO_4 on Chromosorb WAW, 80/100 mesh) with flame ionization detection (15 psi H_2, 25 psi air) was used in the analysis. The carrier gas was nitrogen (45 psi).

Antimicrobial Assay and TLC Analysis. Five- or 10-ml aliquots were taken periodically during the incubation and 2.1 M citric acid was added (0.1 ml/1-ml aliquot) to terminate the reaction. Acetone was added (equivalent to aliquot volume) and the samples were sonicated for 10 min, shaken for 60 min, allowed to stand for 25 min, and centrifuged at 5000*g* for 5 min. For microbiological assay, the supernatant was diluted with control rumen fluid extract so that the theoretical virginiamycin

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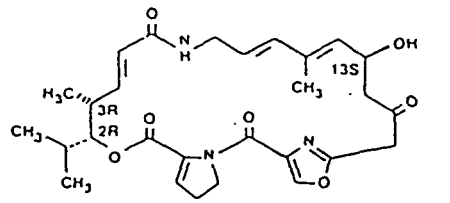
[1] Abbreviations used are: VFA, volatile fatty acid; FAB, fast atom bombardment.



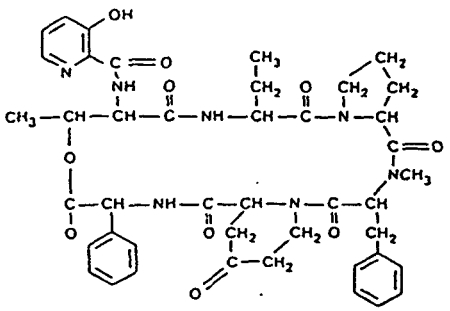


FIG. 1. *Chemical structures of virginiamycin factors M_1 and S_1.*

concentration was 3.12 μg (activity)/ml. Early time-course samples (before 24 hr) were processed to this point and kept at 0°C until extraction of the 24-hr sample was completed.

The resulting solutions were plated against a virginiamycin standard curve (0.64–3.12 μg (activity)/ml) using *Sarcina lutea* as the test organism. The agar plates were incubated overnight and the zones of inhibition were read using a Fisher-Lilly zone reader. The final results (VAX program) were expressed as a percentage of initial activity.

For TLC analysis, the entire sample (initial 5.0-ml aliquot) was diluted to 40 ml with distilled H_2O and applied to a prewashed (methanol, water) C-18 Bond Elut column (6 ml; Analytichem International, Harbor City, CA) and the initial eluent as well as a 5.0-ml wash with 10% acetonitrile/water was collected as fraction 1 (total volume, 45 ml). The column was further eluted with 5.0 ml of 100% acetonitrile, which was collected as fraction 2. Aliquots of fraction 1 (3 × 1.0 ml) and fraction 2 (3 × 0.1 ml) were counted in 10 ml of Atomlight for radioactivity recovery determinations. The remaining 100% acetonitrile fraction was evaporated to a small volume, spotted on a Baker 250-μm, 250F, 20 × 20 cm silica TLC plate, and developed in 93:7 chloroform/methanol. The plate was air dried and analyzed using a Bioscan 100 TLC radioactivity scanner.

Virginiamycin Stability in Aqueous Buffer. A 0.5-ml aliquot of the [^{14}C]virginiamycin stock solution (2.46 mg/ml) in ethanol was placed in a 250-ml Erlenmeyer flask. Immediately afterwards, 99.5 ml of a 0.1 M sodium phosphate buffer, pH 6.5, were added, followed by vigorous swirling for 2 min. The flask was gassed with carbon dioxide for 10 sec, stoppered, and placed in a shaking water bath (Precision model 50) at 39°C, oscillating 100 times per min. After 0, 1, 2, 4, 8, and 24 hr, three 2.0-ml aliquots were removed and analyzed by TLC and for antimicrobial activity as for the rumen extracts (no centrifugation required).

Isolation of Factor M Decomposition Products. [^{14}C]Factor M (61.098 mg; 0.5 μCi) was weighed into a 250-ml Erlenmeyer flask and dissolved in ethanol (5.0 ml) and 0.1 M KH_2PO_4 buffer, pH 6.5 (100 ml). The flask was stoppered, sealed with Parafilm, and placed in a 40°C shaking water bath for 5 days. The sample was lyophilized and 75 ml of water was added to the dried material. The aqueous sample was transferred to a separatory funnel and extracted with methylene chloride (3 × 75 ml). The organic layer was separated, concentrated using a rotary evaporator, and streaked across the bottom of an Analtech Uniplate Taper Plate (silica gel GF). The plate was developed in chloroform/methanol (93:7) and scanned using the Bioscan radioactivity analyzer. The region corresponding to R_F 0.51–0.70 (between factors M and S) was scraped and extracted with methanol (3 × 20 ml). The extract was evaporated to dryness and redissolved in 2% methanol/$HCCl_3$ for HPLC analysis.

The HPLC equipment included an Altex 110 and 100A pump, a Beckman 421 controller, and a Perkin-Elmer LC-55 UV Detector (254 nm) connected to a Hewlett-Packard 5880A Integrator. A guard column containing 10-μm SI-60 packing material (30 × 4.6 mm; Brownlee Industries, Santa Clara, CA) was placed before the Lichrosorb SI-60 analytical column (5 μm, 250 × 4.6 mm; ES Industries, Marlton, NJ). The mobile phase (2% methanol/$HCCl_3$) was pumped at a flow rate of 1.0 ml/min. Two products were collected (R_T 6.2 and 7.5 min) by repetitive HPLC injections (100 μl) of the crude sample scraped from the TLC plate. The products were repurified by reinjection on the same column with the resulting fractions being collected in vials kept on dry ice and maintained under those conditions (in solution) until mass spectral analysis.

Isolation of Virginiamycin Metabolites. Freshly collected cattle rumen fluid was prepared as described above, incubated (39°C) with [^{14}C] virginiamycin for 48 hr, and frozen if not analyzed immediately. Thawed samples were mixed well and 5.0 ml of 2.1 M citric acid were added. The sample was sonicated for 10 min, followed by acetone addition in an amount equal to the volume of the remaining rumen fluid. The sample was shaken for 30 min and centrifuged in an RC2B centrifuge (Sorvall, Division of Dupont, Wilmington, DE) for 10 min at 5000 rpm. The supernatant was decanted and the extraction was repeated two additional times. The combined supernatant was diluted 3:1 with water and applied to an Amberlite XAD-2 (20–60 mesh, Aldrich Chemical Co., Milwaukee, WI) column (2.6 cm × 35 cm) preequilibrated in water. The initial effluent, along with a 1-liter water wash, was collected as one fraction, followed by a second fraction generated by elution with 1 liter of methanol. The methanol was evaporated to dryness on a rotary evaporator, redissolved in 100 ml of 85:15 $HCCl_3$/methanol, and applied to a silica gel (Merck, 35–70 mesh, 40Å; Aldrich) column (2.6 cm × 35 cm) preequilibrated in 85:15 $HCCl_3$/methanol. After the sample had settled into the column bed, elution was continued until 1 liter of 85:15 $HCCl_3$/methanol had been collected from the column. The eluent was evaporated to near dryness on a rotary evaporator, transferred to a scintillation vial, evaporated to dryness, and redissolved in 10 ml of chloroform. Five silica Sep-Paks (Waters, Milford, MA) were equilibrated with 15 ml of chloroform and 2.0 ml of the redissolved sample were applied to each column. The Sep Paks were eluted with 5.0 ml of $HCCl_3$ (fraction A), 10 ml of 10% methanol/$HCCl_3$ (fraction B), and 5.0 ml of 100% methanol (fraction C), with like fractions from the five Sep-Paks being combined.

Fraction B, which was found to contain the majority of the eluted radioactivity, was evaporated to approximately 1 ml and applied to an Analtech Uniplate Taper Plate (silica gel GF). The plate was developed in ethyl acetate/methanol/ammonia (100:10:5), dried, and scanned using a Bioscan 100 radioactivity detector. Five regions of radioactivity were scraped from the plate and extracted (3 × 20 ml) with methanol. The R_F values for the five regions were 0.00–0.24 (no. 1), 0.24–0.32 (no. 2), 0.32–0.44 (no. 3), 0.44–0.55 (no. 4), and 0.55–0.84 (no. 5).

The evaporated extracts from regions 3 and 5 were redissolved in acetonitrile and analyzed by reverse phase HPLC using an Alltech Econosphere 5-μm column (250 × 4.6 mm) with an 18-GU Spheri-10 guard column (10 μm, 30 × 4.6 mm; Brownlee) and the equipment described above.

A gradient program was run from 18.75 to 50% acetonitrile in 0.01 M acetic acid over 15 min, maintained at 50% for 10 min, and taken to 75% acetonitrile in 0.01 M acetic acid over an additional 10 min. The flow rate was also increased from 1.5 ml/min to 2.0 ml/min at 37.5 min after injection. The injection volume was 100 μl with UV detection at 238 nm over a 55 min total run time.

Fractions were collected based on UV absorbance of the eluting peaks and aliquots were taken for counting and quantitation of the radioactivity. Factors M and S and metabolites A, C, and D eluted between 14 and

40 min when chromatographed using the above gradient procedure. Generally, five fractions were collected as shown in table 1 and lyophilized overnight.

Final purification was realized by normal phase HPLC using two Waters model 590 pumps, a Waters model 721 controller, a Waters model 481 λMax UV detector (254 nm), an Autochrom Solvent Select Valve, and a Rheodyne model 7126 injection valve (100 μl loop) equipped with pneumatic activation. The lyophilized fractions (1–5) were redissolved in $HCCl_3$ and chromatographed on a Lichrosorb SI-60 column with an isocratic solvent system of 5% methanol/$HCCl_3$ at a flow rate of 1.0 ml/min.

The metabolites eluted with the following R_T: metabolite D, 5.39 min; metabolite C, 5.48 min; factor M, 5.84 min; and metabolite A, 6.59 min. The collected samples usually required one or two reinjections on the same column in order to remove additional impurities. The purified metabolites were quantitated by counting aliquots for determination of total collected radioactivity. The samples were stored in solution (5% methanol/$HCCl_3$) at −80°C before structural analysis.

Metabolite E (TLC region 4) did not require preliminary purification by reverse phase HPLC. The evaporated TLC extract was redissolved in 5% methanol/$HCCl_3$ and isolated directly by repetitive injection on the normal phase system described above. Metabolite E eluted with a R_T of 5.1 min.

UV Spectroscopy. UV spectra were recorded using either a Gilson model 240 or a Perkin-Elmer lambda array 3840 UV/VIS spectrophotometer equipped with a PE 7300 professional computer and a PE PR-200 printer. UV spectra of the factor M decomposition products were obtained using a Hewlett-Packard 1040 diode array detector in conjunction with a Hewlett-Packard chromatography system, including a model 1090 liquid chromatograph, model 7470A printer, and an HP85B computer interfaced to a model 9121 dual disk drive data system.

Mass Spectrometry. Mass spectral analysis was conducted on a VG 7070E instrument (FAB) using xenon gas at an accelerating voltage of 6 keV. Data analysis was handled by a Digital 11/250 Data System. The sample matrix was either glycerol (molecular weight 92) or thioglycerol (molecular weight 108). Additional FAB spectra, high resolution data, and collision activation analysis (MS-MS) were obtained on a Kratos MS-50TA instrument at the Midwest Center for Mass Spectroscopy in Lincoln, Nebraska. The sample matrix was dithiothreitol (molecular weight 154) with or without added sodium chloride. LC-MS spectra were obtained on a Finnegan 4500 instrument with a Vestec Thermospray interface at the National Institute of Environmental Health Sciences, Research Triangle Park, NC. On-column injections were conducted using an ASI C_{18} column with a gradient from 24.7 to 50.05% acetonitrile/0.1 M ammonium acetate over 20 min at 1.0 ml/min.

NMR. NMR spectra were obtained on either a JEOL GX-270 or GX-500 MHz instrument at Smith Kline & French Research Laboratories. The isolated metabolites were evaporated to dryness, lyophilized overnight to remove traces of residual solvent, and redissolved in DMSO-d_6 for analysis. Data acquisition generally occurred over an 8–24-hr period.

Microbiological Assay of the Metabolites. Metabolites A, C, and D were tested for antimicrobial activity against a factor M standard curve (1–5 μg/ml) using *Corynebacterium xerosis* as the test organism. The metabolite concentrations were determined by counting aliquots of the dissolving solutions for radioactivity. The specific activity of the parent compound (factor M) was used to calculate the metabolite sample weight. The activity results were expressed as a percentage of factor M activity.

TABLE 1

Description of HPLC fractions

TLC Region	HPLC Fraction	Time	Contents
		min	
5	1	14–16	Metabolite A, Factor M
	2	16–18	Factor M, Metabolite A
	3	18–19	Metabolite C
	4	19–21	Metabolite D
	5	21–22	Metabolite D
3	1	35–40	Factor S

Results

VFA Analysis. The production of VFA was used as an assessment of the quality and uniformity of the rumen fluid. Actively fermenting samples showed general increases from approximately 62.3 to 155.1 mmol/liter total VFA over a 24-hr period. The addition of 8–32 ppm (virginiamycin activity) depressed VFA production (microbial activity) by about 20%, an effect that would be expected to occur *in vivo* as well. Based on this finding, the subsequent *in vitro* studies were all conducted at 24 ppm (virginiamycin activity). Although some depression of fermentation would be expected at this level, it was judged to be of limited consequence to the overall virginiamycin activity profile and production of specific metabolites.

Sterilization of the rumen fluid (autoclave, 15 min) before the addition of virginiamycin resulted in the cessation of microbial activity. No increase in VFA production over time was observed for sterilized rumen fluid samples.

Metabolism and Chemical Degradation Studies. A series of experiments were performed to monitor the behavior of virginiamycin during exposure to conditions of (a) pH 6.5 sodium phosphate buffer, (b) normal rumen fluid, and (c) sterilized rumen fluid. In all three instances, aliquots were removed from the incubation flasks at various time points and assayed for antimicrobial activity. A plot of the activity loss *vs.* incubation time is shown in fig. 2. In addition to antimicrobial assays, aliquots were extracted and analyzed by TLC, and the progress of the incubation was followed through the use of a radioscanner. Fig. 3, *A–D* illustrates the degradation and/or metabolism of virginiamycin after incubation under the various conditions for 24 hr.

When [^{14}C]virginiamycin was incubated in pH 6.5 buffer at 39°C, an additional peak that chromatographs between factors M and S was observed (fig. 3*B*). After 24 hr, this new product

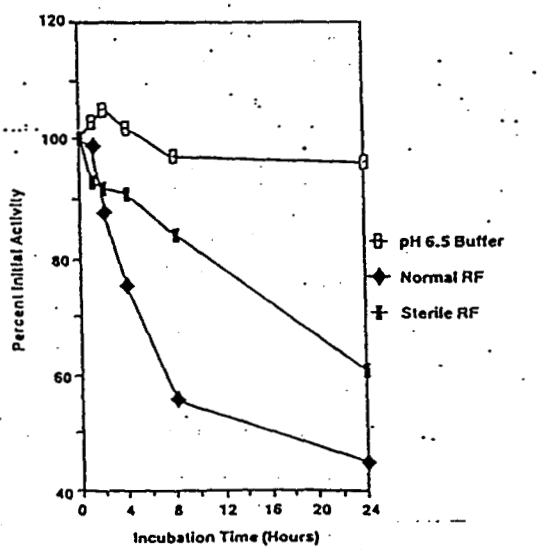


FIG. 2. *Microbiological activity loss of virginiamycin after incubation in pH 6.5 buffer, normal rumen fluid, (RF), or sterile rumen fluid.*

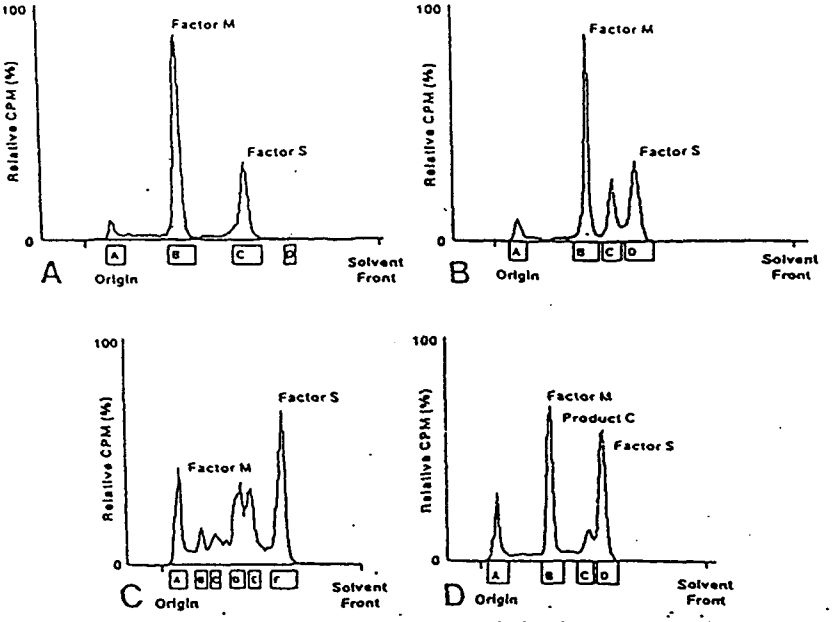


FIG. 3. *TLC radiochromatograms of virginiamycin after incubation in pH 6.5 buffer for 0 hr (A), pH 6.5 buffer for 24 hr (B), normal rumen fluid for 24 hr (C) and sterile rumen fluid for 24 hr (D).*

comprised 19% of the total radioactivity. Inasmuch as the percentage of the total radioactivity attributed to factor M decreased in proportion to the increase observed in the new peak, it suggested that the new product was derived from factor M. The HPLC chromatogram generated during the last phase of isolation (see *Methods*) indicated the presence of two compounds, which eluted at approximately 6.2 and 7.5 min, respectively. When these peaks were collected separately and reinjected to check purity, both were still present in each sample. Because this observation did not change upon repeated isolation attempts, it was suspected that the two compounds were interconverting in solution at room temperature. Repetitive chromatography over a 12-hr period of a solution enriched in either compound verified this interconversion, as the two compounds appeared to attain an equilibrium of 2:1. Successful isolation was finally achieved by collecting the HPLC fractions in vials surrounded by dry ice.

UV spectra of both compounds (designated compound 1 and compound 2) were obtained by diode array scanning of the eluting peaks (fig. 4*A*). Compounds 1 and 2 had λ_{max} values of 330 and 340 nm, respectively, indicating a higher degree of unsaturation than factor M, which has a λ_{max} at 276 nm (fig. 4*B*).

Mass analysis (FAB) of both compounds gave spectra that were identical. In both cases, a molecular ion (M + 1) was observed at 508 mass units, representing a mass loss of 18 ($-H_2O$), inasmuch as factor M has a molecular ion at 526 (M + 1). The data suggest that the two compounds are geometric isomers resulting from the dehydration of factor M at the allylic hydroxyl group.

The radiochromatogram from the 24-hr incubation of [^{14}C] virginiamycin with normal rumen fluid (fig. 3*C*) indicates that a significant amount of metabolism has occurred. Integration of the major regions of radioactivity at each time point suggest that all the metabolites arise at the expense of factor M. The percent-

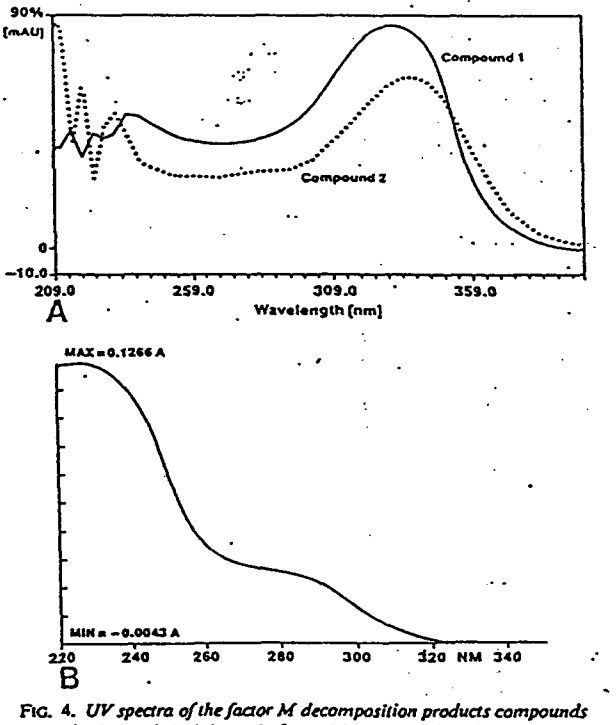


FIG. 4. *UV spectra of the factor M decomposition products compounds 1 and 2 (A) and virginiamycin factor M in 2% methanol/chloroform (B).*

age of the total radioactivity associated with factor M decreased steadily over the incubation period whereas the percentage associated with factor S remained relatively constant. A summary of the TLC data is shown in table 2.

In contrast, the radiochromatogram from the 24-hr incubation of [^{14}C]virginiamycin with sterile rumen fluid (fig. 3*D*) indicates that substantially less metabolism occurs after destruction of the rumen bacteria. The TLC scan does indicate the formation of a new product that apparently is derived from factor M (area integration analogous to table 2). The isolation and identification of this product was not pursued any further at this time, although close examination of the R_F value of the product indicated that it was not identical to product(s) generated in the buffer-only system (fig. 3*B*).

Metabolite Isolation. Generation of sufficient material for characterization required a scale-up of the rumen fluid incubation procedure as described in *Methods*. These changes resulted in the production of larger quantities of the same metabolites, without altering the observed metabolic profile. The overall yield of the isolated metabolites is shown in table 3.

Metabolites A, C, and D were assayed for antimicrobial activity *vs.* a factor M standard curve. The results indicated that metabolite C was 46.4% as active as factor M itself, whereas metabolites A and D were devoid of activity.

Identification of Metabolites A and C. The UV spectra for metabolites A and C were essentially identical to that of factor M (fig. 4*B*) and indicated that no increase in the extent of conjugation had occurred. Preliminary identification of metabolites A and C was achieved by comparison of their FAB MS spectra with the spectrum of factor M. The key features of the factor M spectrum included a molecular ion (M + 1) at mass 526 along with a major fragment at mass 508 (M + 1−18), which indicated a loss of H_2O. This water loss has been observed previously (12) and has been attributed to the dehydration of factor M through loss of the allylic hydroxyl group. Ions were observed at masses 662 and 680, which can be explained as matrix adducts of the 508 and 526 ions (molecular weight of dithiothreitol, 154). The spectrum of metabolite A indicated an intense peak at mass 510, which was attributed to the molecular ion (M + 1). High resolution analysis of the 510 ion resulted in an exact mass of 510.2596 and an empirical formula $C_{28}H_{36}N_3O_6$. This differs from factor M ($C_{28}H_{36}N_3O_7$) through the loss of one oxygen atom. The majority of the other ions in the spectrum were due to the glycerol matrix (molecular weight) 92; ions 185, 277, 369, 461, 553, and 645) or to glycerol adducts of the molecular ion (510 + 92 = 602). The spectrum of metabolite C showed a molecular ion (M + 1) at mass 512. High resolution analysis of the 512 ion resulted in an exact mass of 512.2742 and an empirical formula of $C_{28}N_{34}N_3O_6$. This differs from metabolite A by the addition of two hydrogen atoms, indicating that a reduction has probably occurred. An additional ion due to a matrix adduct (512 + 154 = 666) was also visible. In order to generate ions useful for identification, a FAB MS-MS experiment was conducted with factor M and the two metabolites. The molecular ions (526, 510, and 512) were individually selected for collision activation analysis. These spectra are shown in fig. 5.

The mass spectral data were conclusive for metabolite A and indicated that the allylic hydroxyl group from factor M had been replaced by hydrogen (CH—CHOH—CH_2 → CH—CH_2—CH_2). Several possibilities existed, however, for metabolite C, based on the mass spectral results alone. The data indicated that a two-hydrogen reduction had occurred; however, there were three possible sites of reduction in the region in which the metabolic change had taken place. Specifically, these sites are the two alkene (C = C) groups β and δ to the carbon atom that initially contained the allylic hydroxyl and the carbonyl group (C = O) β to the same position. As a result, NMR was required for definite structural assignment.

The 500 MHz spectrum of factor M (dimethylsulfoxide-d_6) shows a strong singlet at 1.6 ppm, due to the methyl group attached to the alkene carbon β to the carbon bearing the allylic hydroxyl group. This singlet was also present (as expected) in metabolite A but was absent in the spectrum of metabolite C. This fact, plus the observed increase in proton count in the 0.8–1.1 ppm range, indicated that reduction had occurred at the double bond bearing the methyl group.

Identification of Metabolites D and E. The structures of metabolites D and E were assigned by thermospray LC-MS using an ammonium acetate/acetonitrile gradient elution system. The spectra of factor M and these two metabolites are shown in fig. 6. The factor M spectrum again showed the molecular ion at 526 (M + 1) and the ion at 508, which resulted from dehydration. The base peak at mass 262 corresponded to cleavage of the carbon-carbon bond adjacent to the allylic hydroxyl group on one end, and double bond formation *via* McLafferty rearrangement at the lactone group on the opposite end. Thus, mass 262 is diagnostic for the allylic hydroxyl group; this ion was not present in the LC-MS spectra of metabolite A or C.

Metabolite D showed a molecular ion (M + 1) at mass 510 but no 262 ion. Its UV spectrum was different from that of factor M (and metabolites A and C) and had an absorption pattern characteristic of a triene system (13). Additional evidence for the increased conjugation was obtained from NMR, which showed two additional protons in the alkene region. The most likely structure meeting all these criteria would arise through dehydration of the allylic hydroxyl group and subsequent reduction of the carbonyl group adjacent to the newly formed alkene. Metabolite D thus contains an allylic hydroxyl group at an alternate

TABLE 2

Virginiamycin metabolism in cattle rumen fluid: TLC (Bioscan) results summary

Incubation Time	Material at Origin	Factor M	Factor S	Other Metabolites
hr		*% of counts*		
0	8.7	62.9	18.3	
1	8.9	51.2	27.2	
2	6.7	48.3	32.2	3.4
4	9.7	42.9	30.9	3.6
8	11.0	31.3	31.4	10.5
24	13.7	18.7	29.8	25.7

TABLE 3

Radioassay data for extraction of virginiamycin metabolites from cattle rumen fluid: metabolite percentages

Component	Amount Extracted
	% of initial rumen radioactivity
Metabolite A	7.9
Metabolite C	6.1
Metabolite D	3.7
Metabolite E	5.4
Factor M	4.7
Factor S	12.5

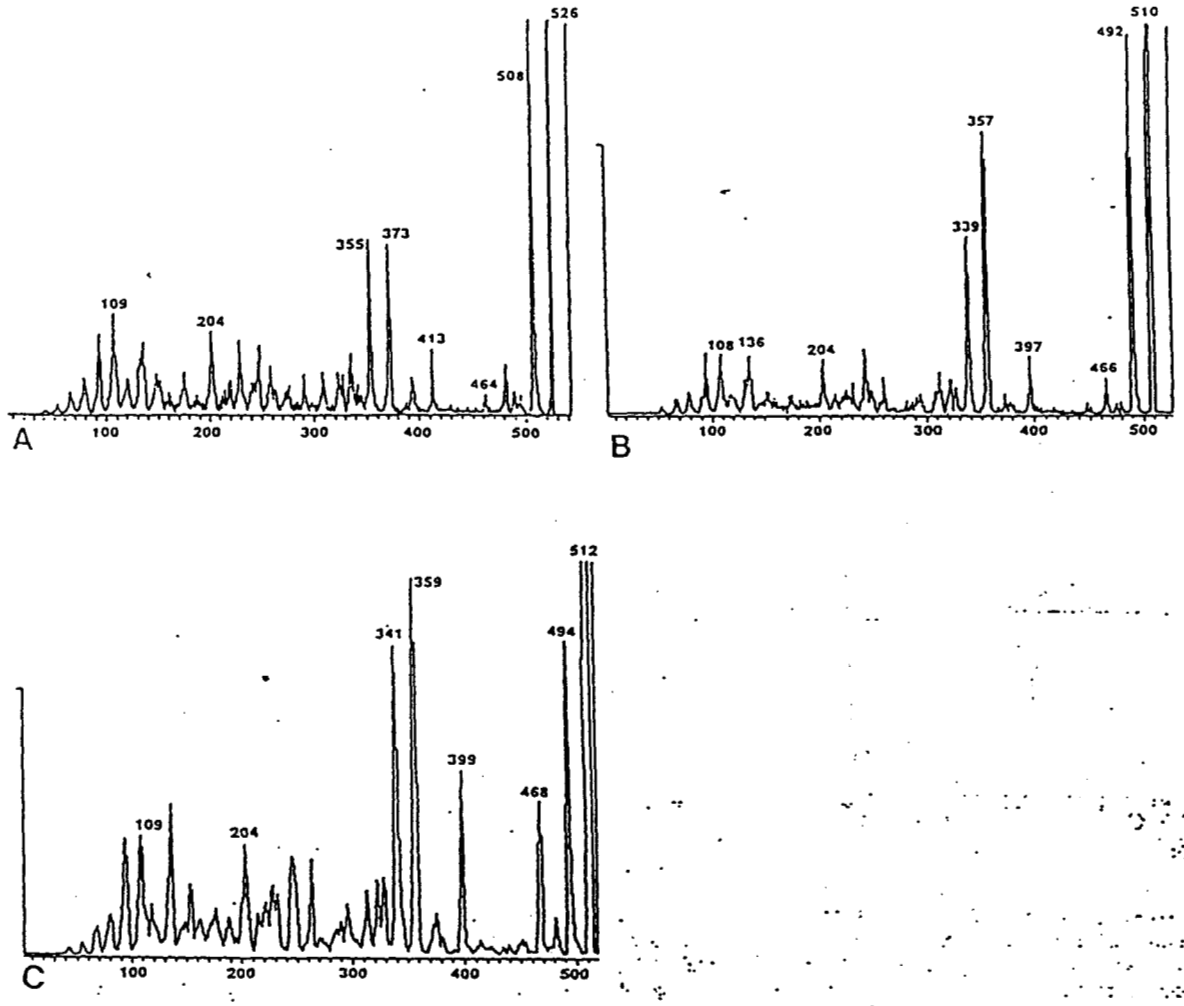


FIG. 5. *Collision activation analysis (FAB MS-MS) of the molecular ions of virginiamycin factor M (A), metabolite A (B), or metabolite C (C).*

location. β-Cleavage at this position, analogous to factor M (generating ion 262), results in the ion at mass 288.

In contrast, metabolite E showed a molecular ion at 528 (M + 1), indicating that a two-hydrogen reduction has taken place. The presence of the 262 ion indicated that the allylic hydroxyl, as well as the two alkene groups comprising that fragment, are still intact. The presence of the ion at 288, however, indicated that the reduction must have occurred at the same carbonyl group as in metabolite D.

Discussion

It is well established that ruminal bacteria have the capacity to perform a variety of biochemical transformations including decarboxylation (14, 15), hydrolysis (16, 17), and reduction (18, 19).

Figs. 2 and 3 demonstrate that virginiamycin is actively metabolized by (normal) cattle rumen fluid. The antimicrobial activity loss occurs rapidly during the early incubation times ($t_{1/2}$ ≈ 8 hr) and becomes slower during the later stages. In sterile rumen fluid, virginiamycin also loses activity but at a slower, more uniform, rate. Inasmuch as fig. 2 indicates that virginiamycin is stable in pH 6.5 buffer alone, the activity loss observed in the sterile rumen fluid ($t_{1/2}$ > 24 hr) is evidence for chemical degradation of the compound (no active metabolism occurs in this situation). The difference in the areas under the normal and sterile rumen fluid curves is a measure of the active metabolism of virginiamycin. At present little is known about this chemical degradation, although virginiamycin is known to lose activity faster in hard water than in soft water and when stored in metal containers as opposed to glass.[2]

The radiochromatograms (fig. 3) correlate well with the antimicrobial activity loss and verify the active metabolism of virginiamycin. The percentage of radioactivity associated with factor M decreases substantially over the incubation period, with concomitant increases in radioactivity associated with the origin and other metabolites (table 2). The factor S percentages remain relatively constant, indicating a metabolically stable compound under these conditions.

[2] SmithKline Beckman Animal Health Products, unpublished data.

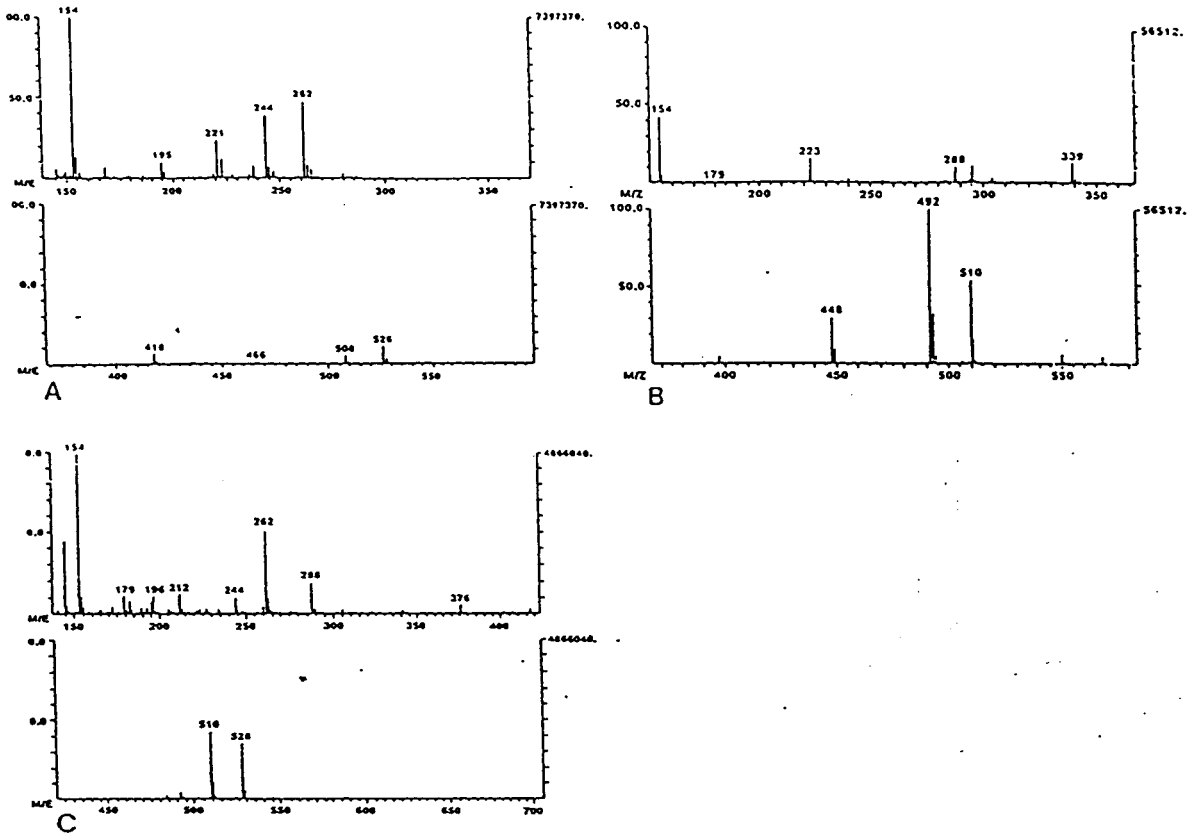


FIG. 6. *LC-MS thermospray analysis virginiamycin factor M (A), metabolite D (B), and metabolite E (C) using an ammonium acetate/acetonitrile gradient.*

The results from the sterile rumen fluid samples reflect a much more stable environment. A single decomposition product forms along with an increase in more polar ^{14}C-derived material, which remains at the TLC plate origin. Once again, the percentage of radioactivity associated with factor M steadily decreases whereas the percentage associated with factor S remains relatively constant. Thus, as with the "other metabolites" (see Table 2) the virginiamycin decomposition product in sterile rumen fluid apparently also arises exclusively from factor M.

The study of virginiamycin stability in buffer at pH 6.5 and 39°C was performed as a control experiment, because these conditions approximate those of the rumen. Fig. 2 indicates that the antimicrobial activity of virginiamycin was stable over a 24-hr period whereas TLC analysis demonstrated the formation of a new product that chromatographs between factors M and S. The discovery that two interconverting structures (compounds 1 and 2) comprise this new product was unexpected but can be explained by loss of the allylic hydroxyl group through dehydration. Loss of H_2O at this position will result in the formation of geometric isomers (*cis/trans*), which can account for the two observed products. These structures are supported by the identical FAB spectra and the increased conjugation evident from UV analysis. The 10-nm difference in λ_{max} values for compounds 1 and 2 is also consistent because *trans*-isomers are known to absorb at higher wavelengths than the corresponding *cis*-isomers (20). Although the mechanism for the interconversion is unknown at this time, it probably involves a rehydration-dehydration sequence of reactions.

Substantial radioactivity losses occurred during the purification and, although four new metabolites were isolated in this study, only about 40% of the total flask radioactivity was accounted for after a 48-hr incubation time (table 3). Nearly 20% of the initial radioactivity remained bound to the rumen fluid pellet and was considered intractable. Additional amounts were lost on the subsequent XAD-2 and silica gel columns, probably due to the presence of highly polar components. The source of radioactivity loss was identified by conducting an experiment in which ^{14}C-labeled factors M and S were incubated separately with freshly collected cattle rumen fluid, according to the procedure described for virginiamycin, and processed through the identical extraction scheme. The radioassay data comparing the extraction of factors M and S clearly indicate that, although factor S is efficiently extracted (100%), a major portion of the factor M radioactivity remained bound to the pellet as an intractable residue. In addition, 87% of the initial radioactivity was recovered in the acetonitrile eluent after Bond Elut isolation of factor S, whereas only 48.1% was recovered in this fraction with factor M. Thus, the source of the recovery losses can be almost entirely attributed to the degradation/metabolism of factor M. No evidence for any significant metabolism of factor S was found in this study.

The four isolated metabolites each comprised less than 8% of

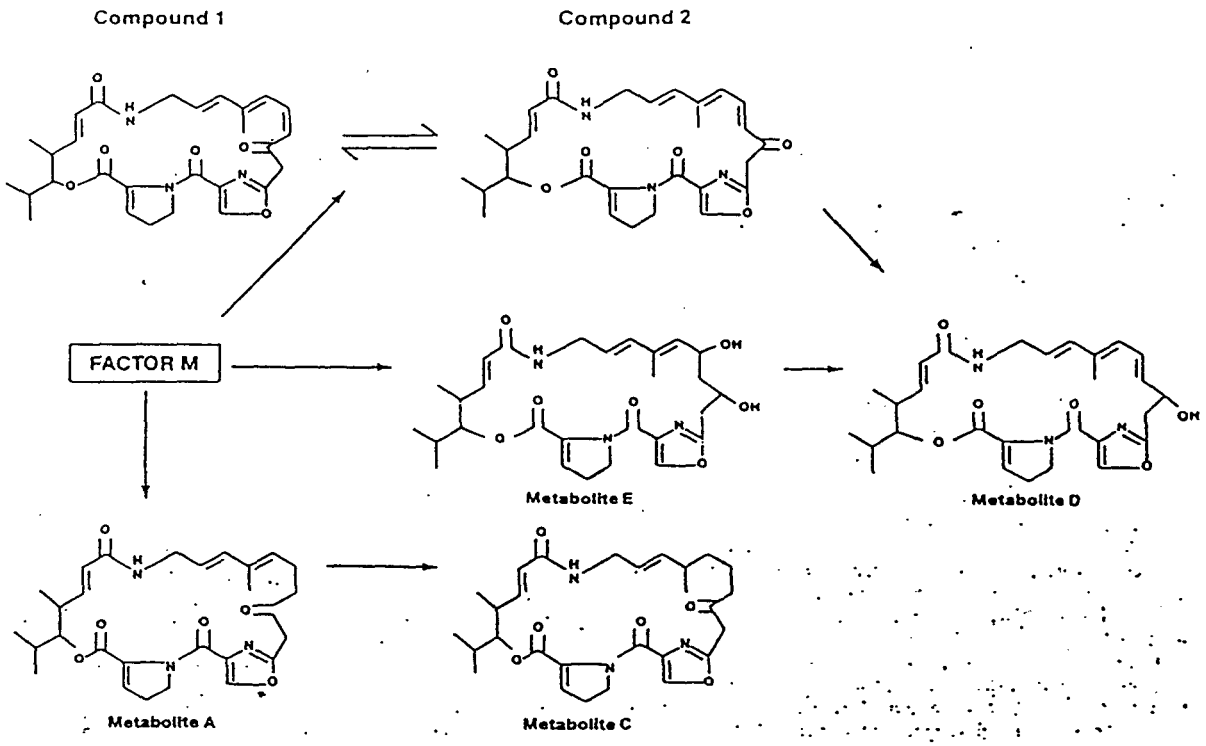


FIG. 7. *Molecular structures and probable metabolic pathway for virginiamycin in cattle rumen fluid in vitro.*

the total radioactivity in the flask after a 48-hr incubation. Metabolite C showed significant antimicrobial activity whereas metabolites A and D were devoid of activity. Metabolite E was not tested. The production of inactive metabolites is, of course, consistent with the activity losses observed in fig. 2.

The FAB MS-MS technique proved essential for structure identification in this study. The resulting scans (fig. 5) not only showed an abundance of fragment ions but were free of the many matrix ions present in the original FAB spectra. Structural assignment of metabolite A was made based on the MS-MS data alone; however, metabolite C (which is a two-hydrogen reduction product of metabolite A) required further analysis by NMR. Due to the limited sample size (100 μg) the spectrum was obtained using a 500 MHz instrument. A major difficulty with this analysis was the presence of water in the sample, even though the isolated material was subjected to extensive lyophilization. When the spectrum was obtained in $CDCl_3$, the water peak (1.8 ppm) obscured the methyl signal of interest at 1.6 ppm (see *Results*). This problem was overcome by substituting dimethylsulfoxide-d_6 for $CDCl_3$, which moved the water peak downfield (3.3 ppm).

In contrast, FAB MS of metabolites D and E was not successful, as no useful molecular weight information was realized. However, these two compounds gave excellent spectra when analyzed by thermospray LC-MS (fig. 6). The fragmentation pattern obtained by this method was clearly unique from that obtained using FAB-MS (compare factor M spectra in figs. 5 and 6) and provided the additional data necessary for structural determination.

The four isolated metabolites are all reduction products of factor M, with the structural changes occurring in the region of the allylic hydroxyl group. Reduction processes were expected because the rumen is essentially an anaerobic environment. Metabolite A results from the dehydroxylation of factor M. The type of reduction is known to occur with intestinal microbes and has been observed in steroid, catecholamine, and mycotoxin metabolism (21, 22). Chemical hydrogenation of factor M has also been shown to convert the allylic alcohol group to a methylene (23). Metabolite C is also a dehydroxylation product with a further reduction of the adjacent carbon-carbon double bond. The reason this compound has antimicrobial activity whereas metabolite A is inactive is not clear. Metabolites D and E result from reduction of the carbonyl group β to the allylic hydroxyl carbon (E) and subsequent dehydration to form a triene (D).

The metabolic profile for virginiamycin is illustrated in fig. 7. Nothing is known for certain about the sequence of metabolite formation, at this time; however, it is logical to assume that metabolism would proceed along the paths of factor M → metabolite A → metabolite C or factor M → metabolite E → metabolite D. It should be noted that compounds 1 and 2, although undoubtedly formed, were not observed during any of the incubations with cattle rumen fluid. However, metabolite D can also be generated by carbonyl reduction of compound 1 or 2, which may explain their absence.

In summary, although relatively stable in buffer at near neutral pH, virginiamycin is rapidly metabolized by cattle rumen fluid *in vitro*. All of the isolated metabolites resulted from the reduction of factor M whereas no metabolism of factor S was evident. The metabolites all had less antimicrobial activity than the parent compound. It is apparent that additional metabolic (or degradation) pathways exist for factor M, inasmuch as these studies

account for only 40% of the radioactivity present in the initial extract. Additional work will be required if these pathways are to be elucidated.

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Appendix VII

STABILITY OF VIRGINIAMYCIN IN CATTLE MANURE
(A non-definitive study)
Study number: V-3003-89

Introduction

Cattle manure, fortified with virginiamycin (Vm), at a level of 34.8 ppm was assayed for microbiological potency at 0, 1, 3, 5, 7, 10 and 14 days after fortification. The extent of microbiological potency loss was used to determine the fate of virginiamycin in feces with time.

Methods

Fresh manure was collected from non-medicated cattle (#317 and #318) and tested for the presence of virginiamycin, and/or possible assay interference from the fecal matrix itself.

After establishing the integrity of the control feces, 5 g samples of the manure were fortified with virginiamycin at a level of 34.8 ppm. Four replicates were prepared for assay at each of the following time points: 0, 1, 3, 5, 7, 10 and 14 days. The samples were prepared in polypropylene containers (50 mL) with caps.

Containers were stored loosely capped at room temperature for the duration of the study.

At the above cited time intervals, the replicate samples were extracted, diluted and assayed for microbiological potency using a standard disc method.

Results

The results of this study are presented in Table 1. The data is expressed as % of the initial (0 day) microbiological potency.

The control feces (from animal #317 and #318) did not show any activity. The standard curve used for calibration and the results of control feces are given in Tables 2 and 3, respectively.

Table 1

Microbiological Potency Loss for Virginiamycin in Cattle Feces

Sample	Concentration of Vm^{+} (ppm)	% of Initial (0 Day Concen.)
Day 0	34.8	100
1	29.9	86.0
3	19.3	55.5
5	12.5	35.8
7	4.6	13.2
10	1.8^{++}	5.1
14	0.9^{++}	2.6

$^{+}$Corrected for 54.9% extraction efficiency.

++Extrapolated data derived from zone sizes which were below the lowest point of the standard curve.

Conclusions

Virginiamycin when fortified into cattle manure at a level of 34.8 ppm rapidly loses microbiological potency. Under these conditions, only 13.2% of the microbiological activity remained after 7 days. The stability of virginiamycin under field conditions would be expected to yield similar results. By days 10 and 14, the microbiological activity levels were below the lowest point of the standard curve (0.64 µg/mL). The calculated half-life for Vm potency in cattle feces was 2.5 days.

000087

Table 2

Standard Curve

Antibiotic Analysis Report 09:41:54 08-02-1989

Title: V-3003-9 FECES STANDARD CURVE (CW-16562:13.
Storage: NA
Ext. Solv: C. ACID/ACETONE
Organism: C. XEROSIS
Matrix: C. ACID/ACETONE/ETOH
Project: MICROBIOLOICAL POTENCY OF VIRINIAMYCIN
Analyst: D.L.
Date Analyzed: 08/01/89
Date Extracted: 08/01/89
Date Prepared: 08/01/89
Procedure: MICROASSAY-DISC METHOD

Standard Curve Calculation

Stndrd Concen (µg/Ml)	Zone Diameter Values — Ref Std Cntrl	CV	Standards Std	CV	Corr.	# Of Plts	Curve Evaluation Std Val	Resid	% Resid
0.64	17.13	0.022	11.64	0.036	11.733	6	0.6463	0.0063	0.98
0.90	17.36	0.019	13.37	0.024	13.308	6	0.9021	0.0021	0.23
1.28	17.31	0.014	14.91	0.028	14.882	6	1.2591	-0.0209	-1.63
2.00	17.33	0.018	17.09	0.016	17.029	6	1.9837	-0.0163	-0.82
3.12	17.23	0.020	19.18	0.014	19.227	6	3.1596	0.0396	1.27

Slope for Std. Curve = 10.872 X-Intercept = 0.054 R^2 Value= 0.9996
Mean of All Ref Stds = 17.272 Std Dev = 0.3271 CV = 0.019
Regression Equation: Y= 10.872 Log(X) + 13.795
Sample Units: mg/ml

Unknown Sample Data Calculation

Sample Number	Zone Diameter Values — Ref St. Uncor	CV	Unknown Uncor	CV	Corr.	# Of Plts	Antibiotic Computation Concen (µg/Ml)	Factor	Calc Value mg/ml

N.B. Ref.: SS 17810: 21

Table 3

000088

Assay Results of Control Feces (#317 and #318)

"MICROBIOLOGICAL POTENCY OF VIRGINIAMYCIN - FECES
""
""
""
V-3003-9 FECES STANDARD CURVE (CW-16562:13.

C. ACID/ACETONE
NA
C. XEROSIS
08/01/89
08/01/89
08/01/89
D.L.

MICROBIOLOICAL POTENCY OF VIRINIAMYCIN
C. ACID/ACETONE/ETOH
MICROASSAY-DISC METHOD

5						
6	.64					
	17.0	12.0	17.4	11.9	16.8	11.0
	17.2	12.0	17.7	12.3	17.2	11.4
	17.0	11.6	17.2	11.5	16.6	11.2
	17.4	11.4	16.5	11.0	17.0	11.9
	16.9	11.7	17.5	12.2	17.7	11.4
	16.6	11.0	17.1	12.1	17.6	11.9
6	.9					
	17.5	13.6	17.5	13.0	17.2	13.5
	18.0	13.3	16.6	12.9	17.4	13.8
	17.2	13.6	17.7	13.3	17.2	12.8
	17.4	13.6	17.0	12.8	17.0	13.3
	17.2	13.4	17.5	13.5	17.1	13.3
	17.6	13.7	17.7	13.8	17.6	13.5
6	1.28					
	17.2	14.7	17.3	14.7	17.2	15.1
	17.6	14.7	17.0	14.7	17.6	15.1
	17.5	15.0	17.0	14.4	17.1	15.4
	17.1	15.4	17.3	14.1	17.0	14.7
	17.6	14.7	17.8	15.6	17.5	15.3
	17.2	15.4	17.4	15.0	17.1	14.4
6	2					
	17.8	17.0	17.4	17.0	17.2	17.1
	17.3	16.9	17.5	17.5	17.2	17.0
	17.3	17.5	17.1	17.0	16.9	16.9
	17.0	16.4	17.2	17.0	17.4	16.9
	17.1	17.1	17.1	17.4	17.9	17.1
	18.0	17.3	17.5	17.0	17.1	17.5
6	3.12					
	17.3	19.6	17.1	19.4	17.4	19.6
	17.1	18.6	16.6	19.2	17.5	19.3
	17.0	19.1	17.9	19.2	17.2	19.1
	17.0	18.9	17.0	19.5	17.5	19.3
	17.7	19.0	16.7	18.8	17.1	19.4
	17.8	19.0	17.0	19.1	17.3	19.2

"#317 FECES = NEG. #318 FECES = NEG.
"SOIL SAMPLES CF PF FH = NEG. → Study # V-3002-89

N.B. Ref.: SS 17810: 22

BIODEGRADATION POTENTIAL OF VIRGINIAMYCIN IN SOILS
(A non-definitive study)
Study Number: V-3006-89

OBJECTIVE

The purpose of this experiment (conducted at Research Triangle Institute, N. C.) was to determine the biodegradation potential of virginiamycin in a variety of soils using the carbon dioxide evolution method. This study was conducted in accordance with FDA Technical Assistance Document No. 3.12 (1987).

METHODS

The amounts of $^{14}CO_2$ and ^{14}C-volatile products released upon biodegradation of ^{14}C-virginiamycin and ^{14}C-glucose in three soil types, incubated aerobically in the dark, were determined for a period of 64 days. The test systems were 250 mL Erlenmeyer flask, containing 50 g of soil (dry weight). Effluent air from each test system flask was purged through scintillation vials containing scintillator which trapped radiolabeled degradation products. Quantitation of trapped radioactivity was performed by liquid scintillation counting (LSC). A total of thirty-three incubation flasks were used to monitor the biodegradation of ^{14}C-virginiamycin and the reference compound, ^{14}C-glucose, each in three soil types (in quadruplicate).

The physical and chemical characteristics of the soil types employed for this study are summarized below:

					Texture (%)		
Soil Type	Cation Exchange Capacity (mec/100g)	% Wt. Loss on Ignition	pH	Field Moisture Capacity(%) % of dry wt.	Sand	Silt	Clay
California Sandy Silt (CA)	14.5	6.3 (n=2)	8.2±0.7	59 (n=2)	40	48	12
Corn Field Silty Sand (CF)	3.5	2.6 (n=1)	6.3±0.0	34 (n=2)	75	20	5
Plymouth Silty Sand (PM)	7.6	4.1 (n=1)	5.7±0.1	36 (n=2)	70	22	8

Preparation of soil samples:

A stock solution of ^{14}C-virginiamycin was prepared in acetone since virginiamycin has a low water solubility. From this stock solution of ^{14}C-virginiamycin, 0.5 mL, (equivalent to approximately 0.5 mg carbon) was applied directly to 5.0 g (dry weight) soil and the acetone was evaporated until the odor of acetone was no longer detected.

Non-labelled glucose, dissolved in water, supplied the remaining 9.5 mg carbon and also served to remoisturize the entire 50 g dry soil to 60% of field capacity.
After addition of the glucose solution the remaining 45 g (dry weight) of soil was then added to the flasks containing 5 g treated soil and mixed upon addition with a glass rod to provide a uniform distribution of virginiamycin throughout the 50 g of soil. The flasks were then weighed, sealed and reweighed and connected to 250 mL flasks containing distilled water. A solvent control blank was established for each soil type according to the above method; however, only acetone was added to the 5.0 g of soil.

A stock solution of ^{14}C-glucose was prepared by dissolving 5 g non-radiolabeled glucose and 100 µCi ^{14}C-glucose in 100 mL deionized water. The ^{14}C-glucose was incorporated into 5.0 g soil after it had been pretreated with 0.5 mL of an acetone blank as described above. Once the acetone was evaporated, 0.5 mL of the ^{14}C-glucose stock solution and the necessary volume of distilled water required for remoisturizing was added to the incubation flask. The remaining 45 g (dry weight) of soil was then mixed upon addition with a glass rod to provide a uniform distribution of glucose throughout the 50 g of soil. The flasks were weighed, sealed, reweighed and connected to 250 mL flasks containing distilled water.

Triplicate 0.5 mL aliquots of the ^{14}C-virginiamycin and ^{14}C-glucose stock solutions were quantitated by LSC prior to treating the soil.

Each incubation flask was attached to a dedicated sampling train consisting of 6 scintillation vials in series. The first vial was a backflow trap, vials 2 and 3 contained scintillator to trap ^{14}C-volatile organics, vial 4 was another backflow trap and vials 5 and 6 contained a phenethylamine based CO_2 absorber. Flasks were flushed during each sampling period for approximately 10 minutes to capture volatile products and evolved carbon dioxide and the traps were replaced. Radioactivity in the samples was measured by LSC daily for the first 2 days and biweekly thereafter.

Flasks were reweighed periodically to detect moisture loss from the soil. Moisture was replenished as necessary with distilled water.

RESULTS

$^{14}CO_2$ and ^{14}C-volatile organics production for ^{14}C-virginiamycin and ^{14}C-glucose are presented in the table below:

Table 1

Cumulative Production of $^{14}CO_2$ and ^{14}C-Volatile Organics Expressed as Percent of Initial DPM Added (Average of Replicates)

Treatment	Soil Type	Average % $^{14}CO_2$	Non-CO_2	Mass Balance
^{14}C-Virginiamycin	CA	40	1	90
^{14}C-Glucose	CA	46	1	93
^{14}C-Virginiamycin	CF	30	2	92
^{14}C-Glucose	CF	66	0	100
^{14}C-Virginiamycin	PM	25	1	87
^{14}C-Glucose	PM	58	1	93

The $^{14}CO_2$ evolution is indicative of the general pattern of virginiamycin biodegradation in the soil. There is no apparent lag period for biodegradation of virginiamycin in any of the soils under any of the test conditions.

If the rate of biodegradation is first order with respect to concentration, calculated half-lives range from 87 to 173 days, as presented in Table 2.

Table 2

First Order Rate Constants and Half-Lives for Biodegradation of Virginiamycin in Soil

Soil	Condition	K (day^{-1})[a]	t 1/2 (days)
CA	^{14}C Vm + non-labeled glucose	0.008	87
CF	^{14}C Vm + non-labeled glucose	0.006	116
PM	^{14}C Vm + non-labeled glucose	0.004	173

[a] Calculated from $A = A_0e^{-Kt}$

where: A = Applied dpm - cumulative dpm from volatiles ($^{14}CO_2$ plus organic ^{14}C at 64 days)

A_0 = Applied dpm; t = 64 days

Degradation of the reference compound, ^{14}C-glucose, was 46 to 66% and the degradation rate was most rapid within the first 10 days in all soils.

CONCLUSIONS

Based on the results obtained during the study, virginiamycin was found to biodegrade in each soil type tested during the 64 day test period under laboratory conditions. Calculated half-lives ranged from 87 to 173 days for the three soils tested, with first order rate constants of 0.004 to 0.008 day $^{-1}$.

Appendix IX

(VIRGINIAMYCIN) - BIODEGRADATION IN SOILS: SUPPLEMENTAL STUDY

Study Number: V-3005-89

Objective

The purpose of this experiment was to determine the aerobic biodegradation potential of virginiamycin in three soil types with different pH, cationic exchange capacity and organic matter and texture, according to the guidelines given in U.S. FDA Technical Assistance Handbook (TAD 3.12).

Methods

The test system consisted of an Erlenmeyer flask (250 mL) containing soil (50 g) attached to a series of glass scintillation vials to trap radiolabelled volatile degradation products. A total of 27 incubation flasks were used to test the biodegradation of relatively uniformly labelled ^{14}C-virginiamycin (0.05 mg carbon/50 g soil amended with 9.5 mg carbon/as unlabelled glucose/50 g soil) and ^{14}C-glucose (10 mg carbon/50 g soil) each in three soil types in triplicate. A single incubation flask containing untreated soil was used for each soil type as a control. The soils used were: 1) Mississippi Silty Clay Loam (CEC: 10.4 meq/100 g, 0.7% organic matter, pH 5.60), 2) Kansas Silty Clay Loam (CEC 14.4 meq/100 g, 2.3% organic matter, pH 6.1) and 3) Ohio Clay Loam (CEC: 10.1 meq/100 g, 1.8% organic matter, pH 5.4). The amount of $^{14}CO_2$ formed upon biodegradation of ^{14}C-virginiamycin and reference chemical (^{14}C-glucose), incubated aerobically in the dark, was determined for a period of 64 days. A preliminary microbial population count was performed prior to test initiation. Moisture was replenished throughout the study as necessary to maintain approximately 55% of field moisture capacity.

High performance liquid chromatography (HPLC) analysis for virginiamycin parent compound in the incubated soils was performed for samples collected on day 1 and 64 after extraction.

Results

The results of the microbial population: 6.87×10^6 CFU/g for Ohio Clay Loam, 6.70×10^6 CFU/g for Kansas Silty Clay Loam and 9.20×10^6 CFU/g for Mississippi Silty Clay Loam indicated the soils used for the test were viable.

The mean cumulative percentages of $^{14}CO_2$ evolved after 64 days from the soils dosed with virginiamycin were 20.5, 17.6 and 12.4% for Kansas, Ohio and Mississippi soils, respectively. Material balance at the end of 64 days averaged 88.3, 85.8 and 101% for Kansas, Ohio and Mississippi soils, respectively. A mineralization half-life value could not be determined since <50% of the added virginiamycin was converted to

$^{14}CO_2$. The mean cumulative percentages of $^{14}CO_2$ evolved after 64 days from the soils dosed with glucose were 40.7, 51.8 and 44.8% for Kansas, Ohio and Mississippi soils, respectively. Material balance at the end of 64 days averaged 90.7, 103, and 93.0% for the Kansas, Ohio and Mississippi soils, respectively.

Results of HPLC indicated extensive breakdown of virginiamycin in all soil types after 64 days. The results of the analysis of day 64 test samples conducted in duplicate are given in Table I. Significant degradation of virginiamycin occurred with less than 6.4% of Factor M and less than 8.6% of Factor S recovered.

Table I

Results of the HPLC Analysis of Soil Extracts

	Day 7		Day 64	
Soil Type	Factor M % of nominal	Factor S % of nominal	Factor M % of nominal	Factor S % of nominal
Kansas Silty Clay Loam	70.4	74.7	<6.4 <6.3	<6.5 8.6
Ohio Clay Loam	96.8	84.7	<6.3 <6.4	<6.4 <6.4
Mississippi Silty Clay Loam	83.3	84.4	<6.2 <6.2	<6.3 <6.3

The extraction efficiency studies conducted after 1 and 64 days incubation in quality control soil samples indicated that an average of 89.8% of the parent compound could be recovered.

Conclusions

Despite slow mineralization of virginiamycin aerobically in soil, extensive breakdown of the compound into a series of minor components with no single degradation product present at >10% of the initial virginiamycin concentration was observed. Based on these results, a "worst case" half-life for degradation can be assumed as 64 days. However, the actual half-life should be within the 64-day incubation period.

In comparison with glucose, mineralization of virginiamycin occurred at a slower rate. This could be attributed to glucose being an easily accessible carbon source for microorganism compared with virginiamycin and also on the percent carbon of the source which is higher for virginiamycin (64%) than in glucose (40%). However, based on structural "rules of thumb,"[13] it is possible to predict that virginiamycin with amide and ester linkages on the structures of Factors M and S is susceptible to biodegradation.

APPENDIX X

DETERMINATION OF THE ADSORPTION AND DESORPTION OF ^{14}C VIRGINIAMYCIN

Study Number: V-3007-89

OBJECTIVE

The purpose of this study was to determine the rate and extent of adsorption/desorption of virginiamycin to soil. The study was performed at Roy F. Weston, Inc.

METHOD

The study was conducted in accordance with the U.S. FDA Technical Assistance Document No. 3.08 (1987). The experimental portion of the study consisted of three phases; preliminary/screening, kinetics and isotherm experiments. Characteristics of the experimental soils used in this study are presented in the following table.

Table 1

Characteristics of Experimental Soils

Soil #	pH	TOC (%)	TOM (%)	CEC (meq/100g
1	5.3	1.01	1.72	11.1
2	7.2	2.12	3.60	26.4
3	6.9	1.97	3.35	10.8

Preliminary/Screening Experiment

A solution of ^{14}C-virginiamycin in either 0.01M $CaCl_2$ or water was added to each soil type and agitated for 16 hours. The samples were centrifuged and the supernatant assayed for total radioactivity. This procedure was repeated adding fresh 0.01M $CaCl_2$ or water to the soil 2 additional times to test desorption.

Kinetics Experiment

A solution of ^{14}C-virginiamycin in water was added to each soil type, agitated, centrifuged and the supernatants assayed for radioactivity at 5, 9.5, 14, 25 and 29 hours. The results of these assays were subjected to statistical analysis to determine when equilibration had been reached.

Isotherm Experiment

Solutions of ^{14}C-virginiamycin in water were prepared at four nominal test concentrations, added to each soil type, agitated for 29 hours, centrifuged and the supernatants assayed for total radioactivity. The soil pellets were then extracted with methanol for 1 hour, centrifuged and the supernatants assayed.

RESULTS

Preliminary/Screening Experiments

Data derived from the preliminary experiments indicated that the analytical method (radioassay by liquid scintillation counting) was sufficiently sensitive to detect the test substance. Results from the screening experiment demonstrated that there was no significant difference in ^{14}C-virginiamycin partitioning between 0.01M $CaCl_2$ and water. Consequently, the kinetics and isotherm experiments were conducted using water as the solvent due to the greater solubility of the compound in water.

Kinetics Experiment

Data from the kinetics experiment indicated that equilibrium partitioning was achieved after 29 hours of agitation for all three soil types. Equilibrium was defined as an overlap in the intervals represented by the mean supernatant concentration + or - one standard deviation for two consecutive samplings. Results are summarized in the following table.

Table 2

Estimated K_d and K_{oc} Values for ^{14}C-Virginiamycin

Sample	pH	Percent Adsorbed	Std Dev.	Est. K_d	Std. Dev.	Est. K_{oc}	Std. Dev.
Soil #1	5.3	57.9	1.9	6.9	0.5	683	50
Soil #2	7.2	68.4	0.9	10.8	0.4	509	19
Soil #3	6.9	72.7	0.2	13.3	0.1	675	5

Isotherm Experiment

For the isotherm experiment, a mean K_{oc} value of 613.1 (std dev = 87.0, % RSD = 14.2%) was derived for ^{14}C-virginiamycin from the data. Mass balances for ^{14}C Vm/soil containing vessels ranged from 56.1 to 68.3 percent; values for ^{14}C Vm soil-less vessels (controls) ranged from 101.8 to 103.8 percent.

CONCLUSIONS

The low recovery of ^{14}C-virginiamycin for the mass balance, was attributed to the poor efficiency of methanol to extract ^{14}C-virginiamycin from soil. Complete recovery of ^{14}C-Vm was observed for soil-less controls, indicating that adsorption to the test vessel was not a factor.

K_{oc} values have been used to estimate the leaching potential of chemicals. In comparison with chemicals of known mobility, virginiamycin may be ranked between moderately to tightly adsorbed to soil.*

* Hamaker, J.W., 1975, The Interpretation of Soil Leaching Experiments, pp. 115-133, in Environmental Dynamics of Pesticides, R. Hague and V. H. Free - Eds., Plenum Press, New York, N.Y.

GREENHOUSE PHYTOTOXICITY EVALUATIONS OF LITTER FROM VIRGINIAMYCIN-TREATED BROILERS ON SEVEN CROPS

Study Number: 00-V-0005-77

OBJECTIVE

The objective of this project was to determine the effects of litter from virginiamycin-treated poultry on crop growth. The poultry were given feed containing 20 grams per ton of virginiamycin. The litter was incorporated into the covering soil to a depth of 2-1/2 inches at 4 - 10 tons per acre.

METHODS & MATERIALS

During the fall of 1976, SmithKline Animal Health Products collected litter from pens of broilers which were fed a basal ration and a medicated ration containing virginiamycin at 20 grams per ton of feed.

The following were employed in this study:

1. Three separate drums of air-dried medicated poultry litter; approximately 40 kg each.

2. Three separate drums of air-dried control poultry litter; approximately 40 kg each.

3. Five jars of fresh medicated poultry manure.

4. Five jars of fresh control poultry manure.

One-third of each drum was ground in a Hobart food chopper for two minutes and returned to the same drum in a sealed plastic container. The sealed drums were stored at an average temperature of 60°F. Random samples of the ground air-dried manure were submitted for proximate analysis along with the fresh samples for moisture determinations.

Soil for the project was obtained from Wipperfurth & Endres, Waunakee, WI 53597. During 1976, wheat was grown on the soil. The two previous years, the soil was used for growing lima beans. A representative sample of the soil was sent to the state soil lab for analysis and type determination.

The soil was sifted through a 0.5 cm mesh screen and put in 2.25 square foot flats in the greenhouse. The litter application rates for the seven crops were based on the recommendations as presented by the following publications:

University of Maryland Fact Sheet 39
Poultry Manure is Valuable Fertilizer
V.A. Bondel, C.S. Shaffner and H.A. Hunter
Depts. of Agronomy, Poultry and Agronomy
Revised, May 1966

University of Georgia Leaflet 206
Poultry Waste - Georgia's 30 Million Dollar Forgotten Crop
Harry D. Muller, Extension Poultry Scientist
November, 1974

The dosage rate for 1 ton per acre is 46.7 grams per flat. All dosages were calculated on the basis of moisture determinations made immediately prior to the start of the experiment.

For both control and medicated poultry manure, an application rate of 4 tons per acre was used for barley, fescue, wheat, green beans and peppers; 5 tons per acre was used for cucumbers and 10 tons per acre for corn.

The previously ground manure samples were weighed in amounts appropriate for the respective flats. Two and one-half inches of the covering soil of each flat was placed in a 5 gallon container and mixed with the sample for four minutes with a Hobart blender. The flats were tagged with a marker as the mixes were completed.

The treated and untreated flats were placed on the greenhouse bench and seeded. A planting form was used which contained 20 holes equidistant from the flat sides and from each other. The crop, variety, number of seeds per flat, and planting depth were as follows:

Crop	Variety	Seeds per Flat	Planting Depth (cm)
Corn	Wis. 900	20	2.54
Cucumber	Improved	20	1.27
	Chicago		
	Pickling		
Green Bean	Green	20	2.54
	Podded		
	Bush		
Pepper	California	20	1.27
	Wonder 357		
Wheat	Timwin	40	2.54
Barley	Dickson	40	2.54
Fescue	Pennlawn	100	1.27

Immediately after planting, each flat was watered with 2 liters using a sprinkler head to evenly distribute the moisture. Equal moisture per flat was added daily as required.

METHODS

Barley - At 23 days after planting, the total stand count in each plot was recorded. At the same time, the average heights of ten plants in the poultry medicated and poultry control plots and twenty plants in the untreated plots were recorded. In each plot, the readings were taken for the first two plants in row one; plants two, three and four in rows two and three; and the last two

plants in row four. In those instances where no plants or one plant was present, it was so noted.

Wheat - At 22 days after planting, the total stand count in each plot for the wheat was recorded. At the same time, the average heights of two plants per ten locations per plot were recorded. In each plot, the same readings were taken as for barley.

Fescue - At 33 days, a stand count of plants for each of five rows in a plot were recorded and totaled. All plants from each plot were cut 3.7 centimeters from the soil surface, and the weight for each plot was recorded. At 53 days, all plants from each plot were cut 2.54 centimeters from the soil surface, and the weight for each plot was recorded.

Corn - At 22 days after planting, the total stand count for corn was recorded. The heights of the plants per plot were recorded accordingly: 0 - 15, 15 - 30, and 30+ centimeters. Those plants which exhibited wilting of the new growth were recorded for each plot.

Green Beans - At 22 days after planting, the total stand count for green beans was recorded. In addition, those plants with primary leaves at least 5 centimeters wide and 8 centimeters long or longer were recorded, as well as all those seedlings which were smaller.

The weights of all larger bean plants per plot were recorded and the average weight of those plants with leaves at least 5 centimeters wide and 8 centimeters long noted.

Cucumber - At 34 days after planting, the total stand count for cucumbers was recorded. In addition, the heights of the plants per plot were recorded accordingly: 0 - 15, 15 - 30, and 30 + centimeters.

All cucumber plants 15 cm or larger were cut at the soil level, and the weight for each plot was recorded. The roots were removed for observation.

The degree of plant injury was noted, and the number of leaves with necrotic lesions was recorded.

Pepper and Tomato - At 19 days after the pepper had been seeded 10 (6 - 8 centimeters) Stokesdale tomato seedlings were transplanted in each flat.

At 56 and 42 days after transplanting, the number, size, and phytotoxic effects were observed and recorded for the pepper and tomato seedlings, respectively.

RESULTS AND CONCLUSION

Litter from poultry fed with virginiamycin treated feed (20 grams per ton) had no effect on the growth of wheat, pepper, tomato, barley and fescue when applied to loam soil at 4 tons per acre and no effect on corn at 10 tons per acre.

The growth of beans and cucumbers was somewhat inhibited. The number of large bean plants was about 15% less than the controls at 4 tons per acre and the number of large cucumber plants about 20% less at 5 tons per acre. However, no phytotoxic symptoms were observed on any plants.

TROUT AND BLUEGILL SUNFISH ACUTE TOXICITY STUDY WITH VIRGINIAMYCIN

OBJECTIVE

To determine the acute toxicity of virginiamycin in trout and bluegill sunfish.

METHOD

The study was conducted prior to implementation of FDA GLP guidelines (prior to 1979). However, scientifically valid protocols available at the time were used to conduct the study and interpret results.

The protocol for bioassay techniques was in accordance with the Environmental Protection Agency Fish-Pesticide Acute Toxicity Test Guideline. Statistical analyses followed the procedure of Lithfield, J.T., Jr. and Wilcoxon, F. entitled, "A Simplified Method of Evaluating Dose-Effect Experiments", J. Pharm. and Exp. Therap. 96:99-113 (May and August) 1949.

RESULTS

Rainbow Trout

24 hours: LC_{50} = 430 ppm
48 hours: LC_{50} = between 225 ppm and 338 ppm
96 hours: LC_{50} = between 225 ppm and 338 ppm

Bluegill Sunfish

24 hours: LC_{50} = 252 ppm
48 hours: LC_{50} = 240 ppm
96 hours: LC_{50} = between 225 ppm and 338 ppm

CONCLUSION

Trout and bluegill sunfish were found to have a high tolerance for virginiamycin.

Appendix XIII

ESTIMATE OF THE CONCENTRATION OF VIRGINIAMYCIN IN RUNOFF ADJUSTED FOR SOIL ADSORPTION/DESORPTION

Adsorption:

Using the total cattle excretion data from Section 6C.i. of the environmental assessment for virginiamycin, the runoff is estimated as follows:

27.8 mg/kg feed x 9 kg feed/head/day = 250 mg Vm/head/day
and 250 mg Vm/head/day x 150 days maximum length of medication
= 37,500 mg virginiamycin per animal

Desorption:

Using estimated concentration from Section 6C.ii.b, the total weight of virginiamycin (mg) expected in agricultural soil are calculated as follows:

0.56 mg Vm/kg soil x 4247.55 kg soil = 2,378.63 mg Vm

Parameters:

Runoff - 200 sq ft per animal

37,500 mg virginiamycin per animal

2" rainfall per 200 sq ft

$$\frac{200\ ft^2}{animal} \times 2"\ rainfall \times \frac{1\ ft}{12"} \times \frac{28.317L}{ft^3} = 943.9\ L\ water$$

Agricultural Soil - top 6" of soil available

$200\ ft^2 \times 0.5\ ft = 100\ ft^3$

$100\ ft^3 \times 28.317 L/ft^3 = 2831.7\ L\ soil$

(approximate mean surface density of soil = 1.5 g/cc[8] or 1.5 kg/L

2831.7 L soil x 1.5 kg/L = 4247.55 kg soil

Calculations for Runoff:

The following calculations estimate the maximum runoff (adsorption) concentrations for virginiamycin. All K_d values are from Appendix X, Table 2.

X = concentration of test substance in water.

Total Vm available is 37,500 mg

4247.55 kg soil x 6.9* X = mass of Vm in soil
943.9 L water x 1 X = mass of Vm in water
(4247.55 kg x 6.9 X) + (943.9 L x 1 X) = 30,252.00 X

30,252.00X = 37,500 mg (total Vm available)
X = 1.24 mg/L = concentration of Vm in water
and
1.24 mg/L x 6.9 = 8.56 mg/kg = concentration of Vm in soil

It should be noted that the estimated concentrations given above can be used only in a qualitative fashion since the algorithm used in the calculations is not validated.

* Represents lowest K_d value of 3 soil types tested to present "worst case" scenario.

Appendix XIV

Excretion and Tissue Depletion Studies in Beef Cattle
Treated with ^{14}C-Virginiamycin

Protocol No. V-4027-84

Summary

1. The excretion of radioactivity in the urine and feces of 3 beef cattle (2 male and 1 female), have been studied after oral administration of non-radioactive virginiamycin (1 mg/kg bodyweight/day) for fourteen days followed by a single oral dose of ^{14}C-virginiamycin (1 mg/kg bodyweight), which corresponds to 2 mg (activity)/kg bodyweight/day.

2. Concentrations of radioactivity in the tissues of cattle have been measured after oral administration of non-radioactive virginiamycin (1 mg/kg bodyweight/day) for fourteen days followed by oral doses of ^{14}C-virginiamycin (1 mg/kg bodyweight/day) for seven days.

3. After oral doses of virginiamycin for fourteen days followed by a single oral dose of ^{14}C-virginiamycin, totals of 76.12%, 93.62% and 95.50% of the radioactivity was recovered in the urine and feces of cattle #22, #23 and #26 respectively during 7 days. Only 0.73%, 1.38% and 1.27% of the radioactivity was recovered in the urine of cattle #22, #23 and #26 respectively and most was eliminated within 48 hours after the radioactive dose. The high fecal excretion (75.39-94.23%) was slower but mostly complete within 96 or 108 hours.

4. After oral doses of virginiamycin for fourteen days followed by oral doses of ^{14}C-virginiamycin for seven day, tissue concentrations of radioactivity of four groups of three animals sacrificed at 10, 24, 72, and 120 hours after the last radioactive dose, have been measured. Concentrations of radioactivity in muscle and fat were <50 ppb and <250 ppb respectively in all animals. Mean concentrations of radioactivity in the liver were 348 ppb, 386 ppb, 281 ppb and 237 ppb in animals sacrificed at 10, 24, 72 and 120 hours respectively after the last dose. Mean concentrations of radioactivity in kidneys were 399 ppb, 210 ppb, 200 ppb and 112 ppb at 10, 24, 72 and 120 hours respectively after the last dose. Mean concentrations of radioactivity in the bile of animals sacrificed at 24, 72 and 120 hours were 1300, 240 and 50 ppb respectively.

5. Microbiological assays for virginiamycin in cattle tissue samples were performed at SmithKline -RIT, Belgium. Virginiamycin residue levels were non-measurable in all tissues except two, which were only slightly above the sensitivity of the method (25 ppb (weight) or 50 ng (activity)/g).