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Tissue Residue Studies in Swine Treated with Virginiamycin

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Virginiamycin was administered as a feed supplement to swine at a level of 170.5 ppm (155 g/ton) for a period of 18 weeks. Residues of the antibiotic greater than 0.1 ppm could not be detected in muscle, liver, kidney, fat, and skin, even in animals not subjected to a withdrawal period.

Virginiamycin is an antibiotic produced by a mutant of *Streptomyces virginiae,* exhibiting activity *in vitro* and *in vivo* primarily against gram-positive organisms (Van Dijck, 1969). Its biological and chemical properties resemble those of other complex antibiotics, such as mikamycin, ostreogrycin, PA 114 factor, pristinamycin, streptogramin, and vernamycin. These antibiotics have in common the presence of two factors which exhibit separate biological activities against several microorganisms but synergistic activity against a single bacterium (Vazquez, 1966).

Factor M, present in the highest concentration, is a macrocyclic lactone containing an oxazole ring (Figure 1). It strongly resembles ostreogrycin A (Delpierre *et al.,* 1966; Vanderhaeghe *et al.,* 1957). Factor S, the minor component, consists of a cyclopeptide lactone ring (Vanderhaeghe and Parmentier, 1960) (Figure 2) which is similar to the B factor of the ostreogrycin complex (Eastwood *et al.,* 1960) and the I_A component of pristinamycin (Jolles *et al.,* 1965).

Both factors M and S exhibit bacteriostatic activity separately but in combination, while exhibiting their synergistic effect, are bactericidal. Apparently, the primary target for both factors of virginiamycin is the process of translation in which inhibition of peptide chain formation occurs, thereby accounting for their synergistic action (Cocito, 1969).

To date, the primary application of virginiamycin has been its use as a feed additive for the growth promotion of swine and for the prophylaxis and treatment of swine dysentery. In this respect, it is an ideal agent, since the gram-positive nature of this antibiotic avoids the problems of resistance transfer through R factor.

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Its growth promotant effects have been reported on by a number of investigators. In one study, virginiamycin at levels of 44 ppm (20 mg/lb) in feed resulted in significant increases in average daily gains in swine (Jones and Pond, 1963). Griffin *et al.* (1961) reported on the use of virginiamycin in feeds at levels of 22–44 ppm (20–40 g/ton). Again, the results demonstrated significant increases in weight gains of swine. Numerous other investigations confirm the activity of virginiamycin as a growth promotant (Barnhart *et al.,* 1960; Griffin and Lidvall, 1962; Miller and Barnhart, 1961).

Reports from abroad first disclosed the activity of virginiamycin in young pigs against blood scours following artificial challenge with swine dysentery in controlled studies (Scholtz and Philip, 1970). Most recently, virginiamycin was shown to protect young pigs effectively against an artificial challenge of the virulent strain of swine dysentery (Miller *et al.,* 1972). In that study, virginiamycin and tylosin were administered to pigs at levels of 28, 55, and 110 ppm (25, 50, and 100 g/ton) in feed. Virginiamycin was found to be more effective than tylosin at all dose levels in decreasing the incidence of diarrhea and death loss, while weight gains and feed efficiency were all improved.

Recently, we performed studies with virginiamycin in swine to determine its potential for the production of tissue residues under practical conditions. These studies are presented in detail in the following report.

MATERIALS AND METHODS

Chemicals and Supplies. Standard plastic petri dishes having an inside diameter of 100 mm and height of 15 mm with plane bottoms were employed. Stainless steel cylinders with internal diameters of 6.35 mm and heights of 10.0 mm were purchased from J. L. Behmer, Inc., Philadelphia, Pa. Trypticase-soy agar, BBL No. 11043, and trypticase-soy broth, BBL No. 11768, were employed in the assay procedure. All reagents employed were Fisher,

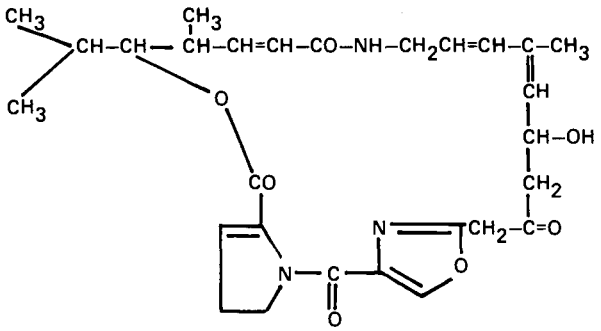

Figure 1. Factor M of virginiamycin.

ACS certified. The assay organism was *Corynebacterium xerosis* NCTC 9755. Virginiamycin standard was obtained from Recherche et Industrie Thérapeutiques, Rixensart, Belgium, in sealed containers and stored at 4°.

Special Instrumentation. Turbidimetric measurements were performed with a Klett-Summerson Model No. 8003 photoelectric colorimeter employing a green filter (540 nm). Zones of inhibition were recorded employing a Fisher-Lilly Antibiotic Zone Reader purchased from Fisher Scientific Company. The Zone Reader was connected to a potentiometer which provided input into a computer punch tape system by means of a Beckman Auto Pro 3111 Intercoupler.

Animal Studies. Six swine (four males, two females) weighing between 10–20 kg were administered virginiamycin incorporated in the feed for a period of 18 weeks. Virginiamycin was incorporated into the swine basal ration as the "Stafac" 500 formulation. Two control animals were maintained on the unmedicated basal ration for the same period of time. Tissues from these animals served as controls for the preparation of the standard curves. The swine basal ration was composed of 66.5% ground shelled corn, 26.5% soybean oil meal, 4.5% dehydrated alfalfa meal, 1.0% trace mineralized salt, 1.0% ground limestone, and 0.5% monosodium phosphate. In addition, vitamins A and D_2 were supplemented at levels of 1000 and 400 units/0.5 kg of feed, respectively. The "Stafac" 500 formulation was composed of 50% virginiamycin (at 100% potency), 30% methyl cellulose, and 20% calcium silicate. Virginiamycin content was based on the standard at 100% potency.

The final medicated feed was assayed at 170.5 ppm (155 g/ton) with homogeneous mixing assured through repetitive assays. This feed represented an exaggerated level of medication, depending on its use, as described in Table I.

Each of the swine was placed in separate stalls and was continuously administered the medicated virginiamycin feed for a period of 18 weeks. Three of the animals were withdrawn from medicated feed at this time and placed on the unmedicated basal ration for 24 hr. They were then sacrificed, along with the remaining three animals, which were not withdrawn from medicated feed. The following tissues were excised and frozen immediately: liver, muscle, kidney, fat, and skin. All of the samples were submitted for assay of virginiamycin content.

Extraction of Virginiamycin from Tissues. Muscle, liver, kidney, and skin (15 g) were cut into small pieces and homogenized in a Waring blender for a total of 4 min with 15 ml of distilled water and 3 ml of 0.2 *M* phosphate buffer at pH 6.1. For skin tissue, twice the volume of water and buffer was required. Each of the tissue homogenates were quantitatively transferred with a total of 175 ml of chloroform to 250-ml graduated cylinders. These were capped and agitated on a horizontal shaker for 15 min and transferred to a 250-ml separatory funnel. Approximately 90–130 ml of the lower chloroform layer was

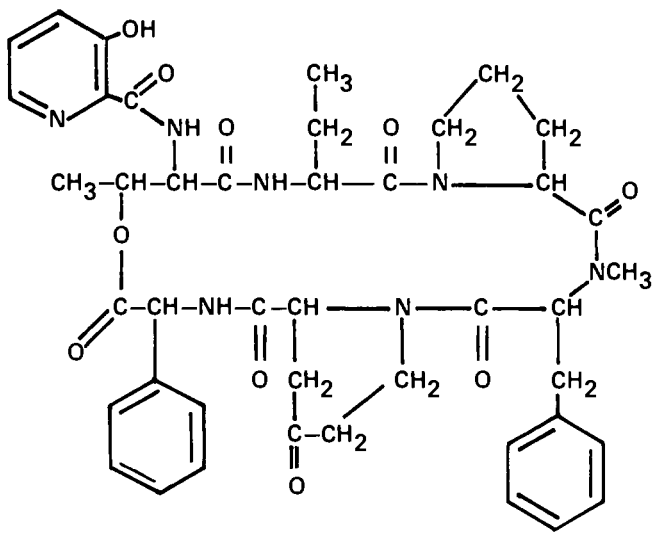

Figure 2. Factor S of virginiamycin.

Table I. Degree of Exaggeration of Virginiamycin Medicated Feed over Various Use Levels

Use	Recommended levels		Percent excess over use levels
	ppm	g/ton	
Prophylaxis	28–55	25–50	84–68
Growth promotion	11–44	10–40	94–74
Therapeutic[a]	110	100	35

[a] Therapeutic levels are employed for 2 weeks, followed by prophylactic regimen.

obtained, and the volume recorded; it was then dried with 15–20 g of anhydrous sodium sulfate for 30 min and filtered through Whatman No. 1 filter paper into a 400-ml beaker. The filtrate was evaporated to dryness in a well-ventilated hood. The residue was redissolved in a minimal volume of acetone and transferred quantitatively to a 10-ml graduated cylinder. The extract was reduced to about 0.75 ml with the aid of a jet stream of nitrogen and adjusted to a final volume of 2.5 ml with 0.2 *M* phosphate buffer at pH 6.1. After mixing well with a vortex mixer, the upper layer of fat globules (which sometimes persisted) was removed by aspiration. The remaining sample was employed directly for the assay of virginiamycin.

Fat samples (15 g) were homogenized with 15 ml of 95% ethyl alcohol. The pulp was removed by centrifugation for 5 min at 1000 × *g*; the extraction process was repeated twice, each time with 15 ml of ethyl alcohol. The resulting residue was quantitatively transferred with a minimal volume of acetone to a 10-ml graduated cylinder and reduced to approximately 0.10 ml with a jet stream of nitrogen. This was diluted with 0.6 ml of ethyl alcohol and adjusted to a total volume of 2.5 ml with 0.2 *M* phosphate buffer at pH 6.1 and assayed for virginiamycin.

Preparation of Assay Inoculum and Medium. A microbial diffusion assay was employed for the detection of virginiamycin in tissues. The assay organism was *Corynebacterium xerosis* NCTC 9755. This organism was maintained on trypticase-soy agar slants prepared by dissolving 40 g of the agar in 1000 ml of distilled water and sterilizing for 15 min at 121°. The organism was transferred daily to fresh slants and incubated at 37° for 24 hr.

In preparing the assay medium, a 24-hr slant of the assay organism was collected in a small volume of trypticase-soy broth. This suspension was then diluted with additional broth until a density of 165 Klett units was obtained on a Klett-Summerson photoelectric colorimeter

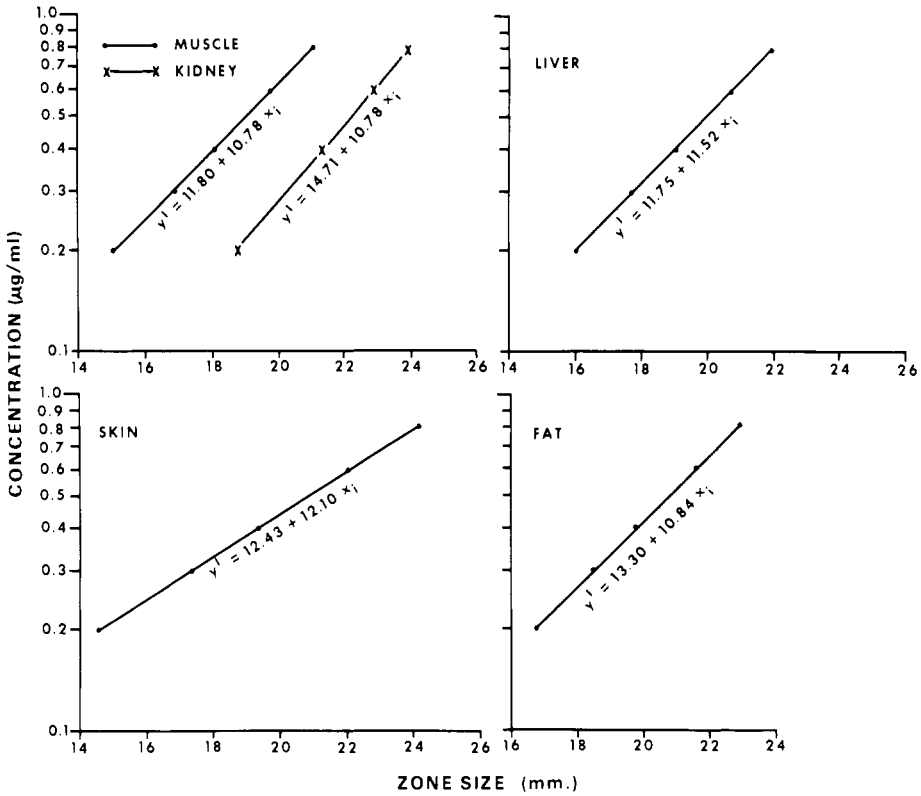

Figure 3. Standard curves employed for assay of virginiamycin in various tissues.

Table II. Intake and Performance Data Obtained from Swine Receiving Feed Medicated with Virginiamycin for a 17-Week Period[a]

Swine no. (sex)	Time of withdrawal	Total feed intake, kg	Average dose of virginiamycin, mg/kg of body weight/day	Weight gain, kg
82 (F)	0	217.9	6.97	56.8
79 (M)	0	228.3	6.96	77.5
81 (M)	0	258.2	6.92	76.9
80 (F)	24 hr	277.9	7.25	78.3
77 (M)	24 hr	291.2	7.24	90.1
78 (M)	24 hr	328.5	9.27	75.1
Average		267.0	7.44	75.8

[a] Data determined for 17-week period only, although animals were medicated for 18 weeks prior to sacrifice.

Table III. Tissue Fortification Studies with Virginiamycin

	Percent recovery from spiked tissues at two concentrations[a]	
	Fortification at 0.05 ppm (±S.D.)	Fortification at 0.1 ppm (±S.D.)
Liver	80 (±3.8)	74 (±5.4)
Kidney	70 (±14.2)	75 (±4.6)
Muscle	63 (±6.2)	87 (±4.7)
Fat	113 (±17.2)	91 (±5.0)[b]
Skin	42 (±13.7)	69 (±5.3)

[a] Average results obtained on four separate tissue samples. [b] Average obtained on three tissue samples.

employing a green filter (540 nm). The final assay medium was prepared by making a 1% suspension of the adjusted inoculum in molten trypticase-soy agar at 45–48°. The agar composition was as follows: 40 g of trypticase-soy agar, 8 g of KH_2PO_4, 2 g of K_2HPO_4, and 1000 ml of distilled water. The pH was found to be between 6.1–6.2 prior to sterilization for 15 min at 121°. Further pH adjustment was not required.

To each of the sterile petri dishes was added 7 ml of the above melted medium containing the seed. Petri dishes were then covered and allowed to harden at room temperature.

Preparation of Standard Curves. Separate standard curves were prepared for each tissue. This was done to eliminate the effects of tissue background on the diffusion of the antibiotic. Extracts of tissues obtained from the undosed control animals were prepared according to the methods previously described. These extracts served as diluent for the preparation of the various concentrations employed for construction of the standard curves.

A 95% ethanolic solution of virginiamycin was prepared at a concentration of 1900 μg/ml (based on the standard at 100% potency). This was the stock solution and could be stored at 4° for 1 month. The stock solution was diluted with 0.2 *M*, pH 6.1 phosphate buffer to a concentration of 5 μg/ml (dilute stock solution). This solution was then diluted with the various control tissue extracts to obtain the standard dilutions necessary for constructing the standard curves, shown as follows: 0.20, 0.30, 0.40, 0.60, and 0.80 μg/ml. These concentrations were selected so that tissue samples containing 0.1 ppm of virginiamycin would result in extracts assaying approximately at the midpoint of the standard curve.

Assay of Samples. For the standard curve, four plates were employed for each of the standard dilutions, which corresponded to 50, 75, 100, 150, and 200% of the reference dilution. The reference dilution was arbitrarily selected at 0.40 μg/ml. On each plate, three cylinders containing the standard dilution were alternated with three cylinders containing the reference dilution (0.40 μg/ml). Each cylinder contained 0.15 ml of sample.

For the assay of unknown sample extracts, four plates per sample were employed. On each plate were alternated

three reference cylinders (containing the reference dilution) with three cylinders containing the unknown sample extract. Each cylinder contained 0.15 ml of sample.

To obtain optimal diffusion, all plates were allowed to preincubate for 60 min at 4°. After preincubation, they were incubated for 18–24 hr at 37° to allow for growth of the organism and subsequent development of the zones of inhibition.

Calculations. Inhibition zone diameters were recorded (to the nearest 0.1 mm) utilizing a Fisher–Lilly Antibiotic Zone Reader. The Zone Reader was modified so that all data were transferred to computer punch tape for processing in a time-share computer system. The following calculations were those programmed into the computer.

Plate to plate variations in zone size were corrected utilizing values obtained for the reference dilution (Stroy, 1969). The standard curve was plotted from the corrected zone diameters by deriving a linear regression line utilizing the method of least squares (Hoel, 1971). Concentrations of unknown samples were then determined from the regression line (standard curve). Standard curves obtained for each of the tissues are presented in Figure 3. A plot of the zone sizes (mm) *vs.* the log of the concentrations results in a linear dose response.

To convert to tissue concentration (in ppm) it was necessary to construct a formula which would take into account the changes in extractant volumes. This formula is ppm = $C(S/Z)$ (2.5 ml/W), where C = concentration of unknown (μg/ml), Z = volume of $CHCl_3$ recovered after extraction, W = weight of tissue sample assayed (g), and S = initial volume of $CHCl_3$ employed for extraction (175 ml). In the case of determinations in fat, the initial volume of solvent was concentrated to dryness and reconstituted to a final volume of 2.5 ml. Therefore, the formula was modified to read ppm = C(2.5 ml/W).

RESULTS AND DISCUSSION

Feed and Drug Intake. Although medicated diet was administered for 18 weeks, weight gain and drug intake data were only recorded for a 17-week period. As indicated in Table II, the average feed intake during the 17-week recording period was 267 kg. This resulted in an average weight gain of 75.8 kg and an average virginiamycin intake of 7.44 mg/kg of body weight/day. This weight gain is consistent with those obtained for animals in an optimum state of nutrition.

Recovery Studies on Fortified Tissues. Recovery of virginiamycin from four replicate samples of muscle, kidney, liver, fat, and skin was performed by fortifying these tissues with the antibiotic at concentrations of 0.05 and 0.10 ppm. These tissues were then extracted and assayed according to the procedures described under the Materials and Methods section. The results of these studies indicate that virginiamycin can be assayed reproducibly in tissues at levels of 0.1 ppm. At 0.05 ppm, recoveries were more variable, although zones of inhibition were still detectable (Table III). These data indicate that the assay procedure is sensitive to virginiamycin in the tissues at levels of 0.1 ppm.

Tissue Residues. Muscle, liver, kidney, fat, and skin samples from the six treatment animals were assayed for virginiamycin content. Four replicate samples were assayed for each tissue. All assays were performed within 10 days of sacrifice, a period for which virginiamycin had been predetermined to be stable in the presence of the analogous tissues. No zones of inhibition could be detected in any of the samples assayed, indicating the absence of significant virginiamycin residues (less than 0.1 ppm). Also, these results strongly suggest the absence of virginiamycin at levels of 0.05 ppm, since the assay method can semiquantitatively detect the antibiotic at this concentration.

It is concluded that the use of virginiamycin as a feed additive in swine results in no detectable residues, even in animals not subjected to a withdrawal period. Its use, therefore, should eliminate the requirement for a withdrawal period and limit its abuse potential under field conditions.

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