Determination of Virginiamycin Residues in Swine Tissue Using High-Performance Liquid Chromatography

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A method was developed for detecting the M_1 factor, the primary constituent of commercial virginiamycin, in animal tissues and blood serum by HPLC with UV detection at 254 nm. Tissue (blood serum) was blended (mixed) with 3 mL of 0.2 M $NH_4H_2PO_4$/g, mixed with an equal volume of methanol, and filtered. After the filtrate was washed with petroleum ether, the virginiamycin was extracted into 3:2 methylene chloride–petroleum ether. Water and acetonitrile were added, and the mixture was evaporated to 1–2 mL under reduced pressure and taken up in 80:20 water–acetonitrile. For analysis, a Supelco LC-18-DB column was used, solvent flow 1 mL/min, with gradient elution [0.01 M $NH_4H_2PO_4$–acetonitrile 80:20 (0–2 min) to 20:80 (25 min)]. Recoveries were near 100% from muscle and serum and 80–90% from liver and kidney with a sensitivity of 10 ppb. Pigs fed 1000 ppm for 1 week had levels of 50–53 ppb in blood serum, 21–26 ppb in liver, 74–79 ppb in kidney, and 41–64 ppb in muscle. Recommended use in feeds at 10–100 ppm should not result in residue problems. The method should be adaptable to determination of virginiamycin in formulations and feeds.

Virginiamycin is an antibiotic complex isolated from a strain of *Streptomyces* resembling *Streptomyces virginianae* (De Somer and Van Dijck, 1955). It consists of two principal components designated M and S. The commerical product contains about 75% of the M_1 and 5% of the S component. The presence of the S component markedly enhances the antimicrobial activity of the M component. Virginiamycin is primarily effective against Gram-positive bacteria (De Somer and Van Dijck, 1955; Vanderhaeghe et al., 1957). The principal commerical use has been as an additive to livestock feeds to promote growth and to control swine dysentery (Leidahl, 1984).

The components of virginiamycin have been separated by paper chromatography (De Somer and Van Dijck, 1955; Vanderhaeghe et al., 1957) and by column and thin-layer chromatography on silica gel (Vanderhaeghe et al., 1957, 1971; Janssen et al., 1977). Separation by reversed-phase HPLC has been reported and used for isolation of the M_1 component, but few details are given (Kingston, 1979; Kingston and Kolpak, 1980). An HPLC method capable of detecting the M_1 and S_1 components in muscle at 0.1 and 0.01 ppm, respectively, using fluorescence detection was recently described by Nagase and Fukamachi (1987). Microbiological procedures have been described both for residues in tissues (Brown, 1985) and for determination in feeds (Ragheb et al., 1979; Mueller-Brennecke et al., 1981; Katz et al., 1984).

De Somer and Van Dijck (1955) found that virginiamycin never attained high tissue concentrations (as demonstrated by biological assay) after parenteral administration. Despite this, it showed excellent activity in mice protection tests. Microbial inhibition was markedly reduced in blood serum or tissue homogenates. The presence in blood and tissue could not be demonstrated after feeding, but some was excreted in the urine, indicating that absorption had taken place. The results suggest that virginiamycin is bound in tissue in a form that is therapeutically effective even though it is not readily detected by microbiological assay. Gottschall et al. (1987) found that less than 10% of virginiamycin fed to cattle or rats was absorbed and that much of the resulting tissue residues was bound in a form that was not readily extractable.

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Although antibiotic residues in animal tissues can usually be detected by inhibition of test bacteria, identification is difficult and no specific test methods are available for many compounds including virginiamycin. The present study was undertaken to develop a specific HPLC method to establish whether or not unidentified microbial inhibitors are virginiamycin and to quantitate residues if present. A reversed-phase HPLC method was developed for separation of the factors of virginiamycin. A method was also developed for extraction of residues from blood serum and tissues and subsequent determination of the M_1 factor by HPLC.

MATERIALS AND METHODS

Chemicals. Methanol, acetonitrile, methylene chloride, and petroleum ether (30–60 °C) were HPLC grades obtained from several sources. Other chemicals were reagent grade.

Standards. Virginiamycin M_1 was obtained from Sigma Chemical Co., St. Louis, MO. Virginiamycin, virginiamycin M, and virginiamycin S standards were obtained from SmithKline Animal Health Products, West Chester, PA. Stock solutions were prepared by dissolving 10 mg of standard in 20 mL of acetonitrile and diluting to 100 mL with water. Working solutions of 0.01 mg/mL were prepared by diluting the stock solution 1:10 with 10% acetonitrile in water.

Equipment: Vortex mixer, separatory funnels with Teflon stopcocks, 15-mL graduated centrifuge tubes, graduated cylinders, funnels (75 mm), coarse fluted filter paper (Schleicher and Schuell No. 588), balance acurate to 0.1 mg, blender, 250-mL glass stopper side-arm flasks.

HPLC Apparatus. A Varian Model 5000 liquid chromatograph was used with a UV-50 variable-wavelength detector and a Valco automatic loop injector with a 200-μL loop. A Supelcosil LC-18-DB column, 5-μm particle size, 4.6 × 150 mm, with matching guard column (Supelco, Bellefonte, PA) was used.

Sample Preparation. *Tissue.* Five grams of tissue was cut into small (1–2 cm) pieces, weighed accurately, and blended with 15 mL of 0.2 M $NH_4H_2PO_4$. Twenty milliliters of methanol was added to the homogenate slowly with stirring. The mixture was filtered through a coarse fluted filter, and 16 mL (equivalent to 2 g of tissue) of clear to slightly opalescent filtrate was collected. The filtrate was transferred to a separatory funnel and washed with

20 mL of petroleum ether which was discarded. The virginiamycin was then extracted into a mixture of 20 mL of petroleum ether and 30 mL of methylene chloride which was transferred to a 250-mL side-arm flask. Water (3 mL) and acetonitrile (15 ml) were added, and the solvent was evaporated to about 1–2 mL in a 40–50 °C water bath under reduced (water pump) pressure. The residue was rinsed into a graduated centrifuge tube with 0.5 mL of acetonitrile and two to three 1-mL portions of water to a final volume of 4.0 mL and mixed by vortexing.

Blood Serum. A 5-mL portion of blood serum was mixed with 15 mL of 0.2 M $NH_4H_2PO_4$ and 20 mL of methanol. The remainder of the procedure was as described for tissue homogenates.

Spiked Samples. The indicated amount of virginiamycin was added to blood serum or chopped tissue and held 20 min before beginning the procedure.

HPLC Procedure. Chromatography of standards was carried out at several UV wavelengths. For residues, the UV detector was set at 254 nm. Two or more 200-μL aliquots of the sample extract were injected with an isocratic flow of 0.01 M $NH_4H_2PO_4$ (A)–acetonitrile (B) (80:20). Two minutes after the final injection, a solvent gradient was started to a final composition of 20:80 A to B 25 min after the last injection. Peak height was linear with concentration. Quantitation was based on comparison of peak height with a 2-μg standard run during the same 4-h period as the sample. For storage, the column was flushed 2 min with water and stored in 10:90 water–acetonitrile. It was quite stable under these conditions.

Treatment of Pigs. Four newly weaned cross-bred meat type pigs weighing approximately 7 kg each were placed two each in separate pens and fed an 18% protein swine starter diet and water ad libitum during the adaptation period. Seven days prior to slaughter, two of the pigs were switched to a diet containing 1012 ppm virginiamycin (Stafac 44 medicated premix; SmithKline Animal Health Products) in the same diet used during the adaptation period. The pigs were killed by electrocution and blood serum, liver, kidney, and muscle (ham) samples collected and stored frozen until analysis.

RESULTS AND DISCUSSION

The retention characteristics of virginiamycin on a reversed-phase (C_{18}) packing were determined in an acetonitrile gradient in buffer (0.01 M $NH_4H_2PO_4$). The components of virginiamycin can be detected by UV absorption at 220 nm since the absorption maxima are at 207 nm for the S component and 216 nm for the M_1 (Vanderhaeghe et al., 1957). The S component also has a small absorption peak at 304 nm that is too weak to be very useful. Virginiamycin is almost insoluble in water or petroleum ether but is readily soluble in polar organic solvents (De Somer and Van Dijck, 1955). A chromatogram of the viriginiamycin standard is shown in Figure 1. As would be expected from the solubility, virginiamycin was rather strongly retained on the reversed-phase packing, requiring >50% acetonitrile for elution. The M_1 component gave a very sharp peak that was off-scale at the sensitivity shown. The S component gave a small peak well separated from the M_1. Several minor components of unknown identify were also present. Figure 2 shows a chromatogram of the S standard. It was free of the M_1 component and was resolved into a major and a minor component. The peaks were not sharp and tailed considerably. Use of a polymeric column or addition of tetramethylammonium chloride to the mobile phase did not improve peak shape. Separation on silica using HPLC was not attempted since results on the reversed-phase packing were satisfactory.

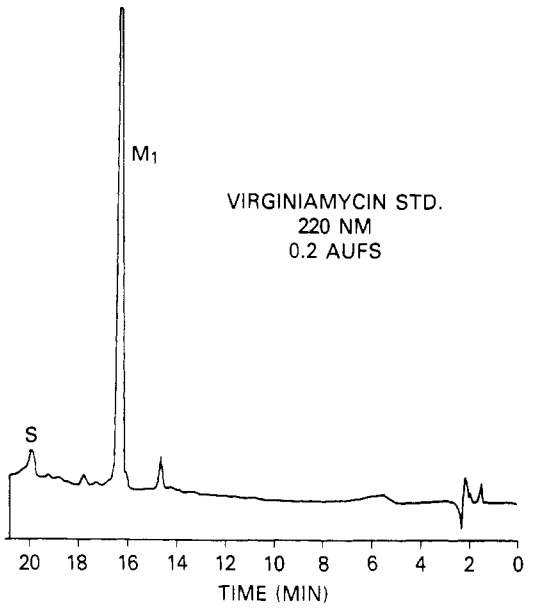

Figure 1. Virginiamycin analytical standard (SmithKline), 2 μg; solvent program, 0.01 M $NH_4H_2PO_4$–acetonitrile, 80:20 (0–2 min) to 20:80 (25 min); injection volume, 200 μL; flow, 1 mL/min; Supelcosil LC-18-DB, 4.6 × 150 mm column.

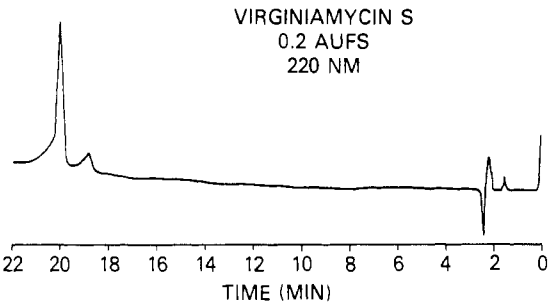

Figure 2. Virginiamycin S standard (SmithKline). Conditions as in Figure 1.

The solvents used in previous studies on silica gel are not suitable for use with UV detection at 220 nm.

Residue analysis was based on the M_1 component since it is not practical to determine minor components separately. Detection was at 254 nm, which gave adequate sensitivity and reduced interference from other substances in sample extracts.

It has been reported that, for analysis of feeds, a proportion of 50% or greater of methanol or other alcohol was required for efficient extraction of virginiamycin (Katz et al., 1984). Addition of various proportions of methanol and acetonitrile to tissue homogenates and blood serum was evaluated. Addition of an equal volume of methanol gave good recoveries of virginiamycin in the filtrate and carried little lipid with it. Higher proportions of either methanol or acetonitrile extracted more lipid without improving recoveries of virginiamycin. However, 1:1 methanol is not very efficient in precipitating proteins. The effects of blending tissue in four different media [water, 0.2 M $NH_4H_2PO_4$, 0.2 M pH 2.2 phosphate buffer, 1 N HCl], all in the proportions 3 mL/g of tissue were compared. The best results were obtained with 0.2 M $NH_4H_2PO_4$. Blending in acid resulted in extraction of a great deal of interfering material, and blending in pH 2.2 buffer gave somewhat lower recoveries.

The filtrate was washed with petroleum ether to remove small amounts of lipid. The viriginiamycin was then partitioned into a methylene chloride–petroleum ether

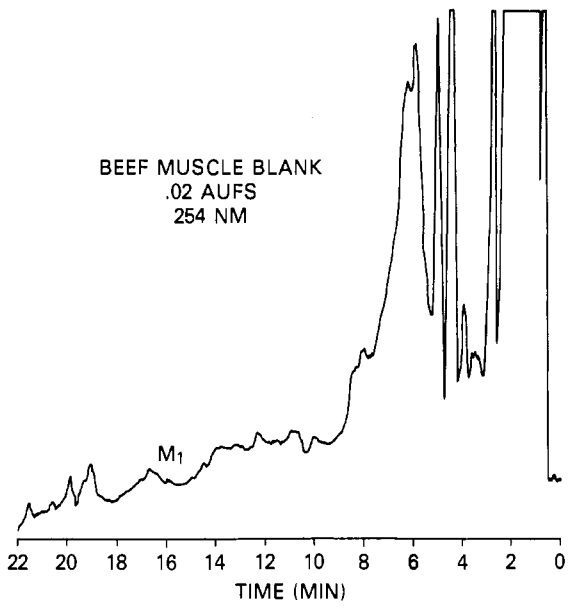

Figure 3. Beef muscle blank, 0.2 g equivalent, 2 × 200 μL injections of sample extract. Conditions as in Figure 1.

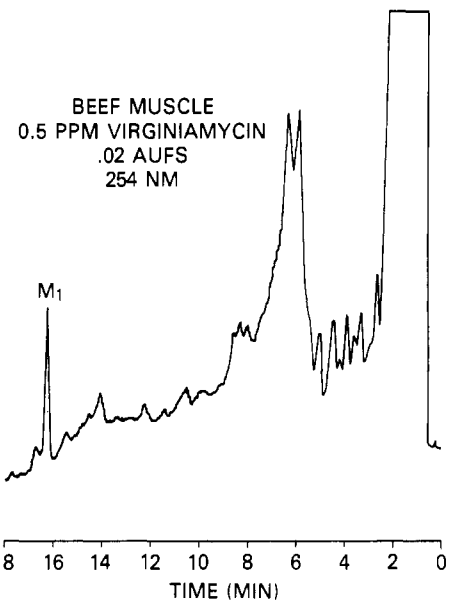

Figure 4. Beef muscle spiked with 0.5 ppm virginiamycin, same as Figure 3.

Table I. Recovery of Virginiamycin from Tissue and Blood Serum

substrate	*N*	amt added	mean recovery ± SD, %
pork muscle	6	0.5–1.0	93.7 ± 3.3
pork kidney	7	0.5–1.0	84.3 ± 4.1
pork liver	5	0.5–1.0	85.8 ± 6.1
beef serum	6	0.5–1.0	98.2 ± 4.2

mixture. Virginiamycin is very soluble in methylene chloride. Petroleum ether was added to reduce carryover of interfering substances.

For HPLC analysis, best results are obtained when the sample is injected in a solvent with little or no eluting strength. Otherwise, chromatographic peaks can be distorted. With a reversed-phase system, the sample must therefore be in a high proportion of water. Transfer of the virginiamycin back to water was accomplished by evaporating the organic layer in the presence of a little acetonitrile and water so that the residue was water. The residue may be concentrated further by evaporation in centrifuge tubes to a small volume. However, we concluded that it was simpler and faster to concentrate the sample on the analytical column by injecting a larger volume of dilute sample with isocratic solvent flow, followed by elution with a solvent gradient. Virginiamycin is immobile on an ODS packing at less than 20% acetonitrile so the solvent composition was adjusted to 80:20 water–MeCN. We used a 200-μL injection loop and made multiple injections to get more sample on the column. A larger loop could be used for single injections of larger volumes of sample. The effectiveness of this method of concentration was previously demonstrated for determination of tetracycline residues (Moats, 1986) and novobiocin residues (Moats and Leskinen, 1988).

The method of Nagase and Fukamachi (1987) has a detection limit of 0.1 ppm for the M_1 component, which is less sensitive than the method described herein. It would not be adequate to detect incurred M_1 residue at the levels found in the present study. Unless the S_1 component is concentrated in tissue relative to the M_1, it also would not be detected at the levels of incurred residue found.

Chromatograms prepared by the multiple-injection procedure are shown for beef muscle. Figure 3 is a blank, and Figure 4 shows the same muscle spiked to 0.5 ppm.

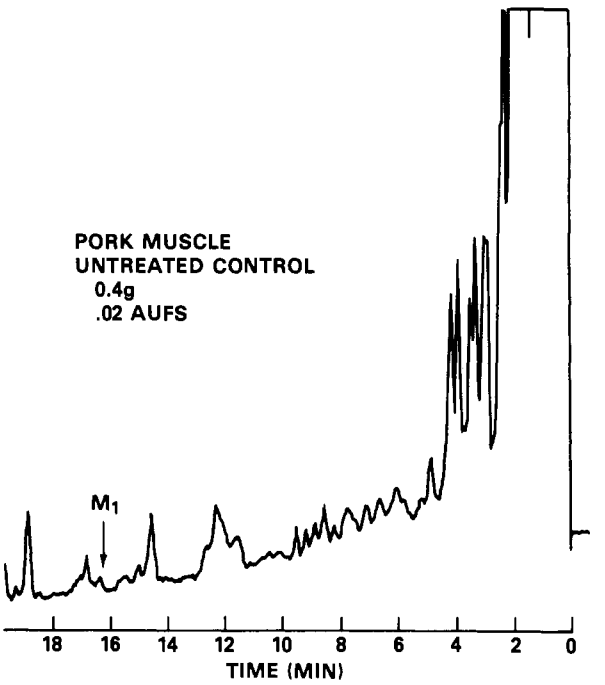

Figure 5. Pork muscle blank, 0.4 g equivalent, 4 × 200 μL injections of sample extract. Conditions as in Figure 1.

Table II. Virginiamycin [ppb (Mean ± SD)] in Blood and Tissues of Pigs Fed 1012 ppm for 1 Week

animal no.	blood serum	muscle (ham)[a]	kidney	liver
101	50 ± 6	64 ± 1	79 ± 1	26 ± 4
102	53 ± 5	41 ± 8	74 ± 4	21 ± 6

[a] Three determinations.

Two 200-μL injections were used. Recoveries are shown in Table I. Recoveries from muscle and blood serum were 90–100% but were somewhat lower from liver and kidney.

In order to determine whether residues could be detected in animals fed virginiamycin, two yound cross-bred pigs (10 kg) were fed a ration containing 1012 ppm of a commerical virginiamycin formulation for 1 week. The animals were then killed by electrocution, and samples of muscle (ham), liver, kidney, and blood serum were collected and immediately frozen prior to analysis. Two

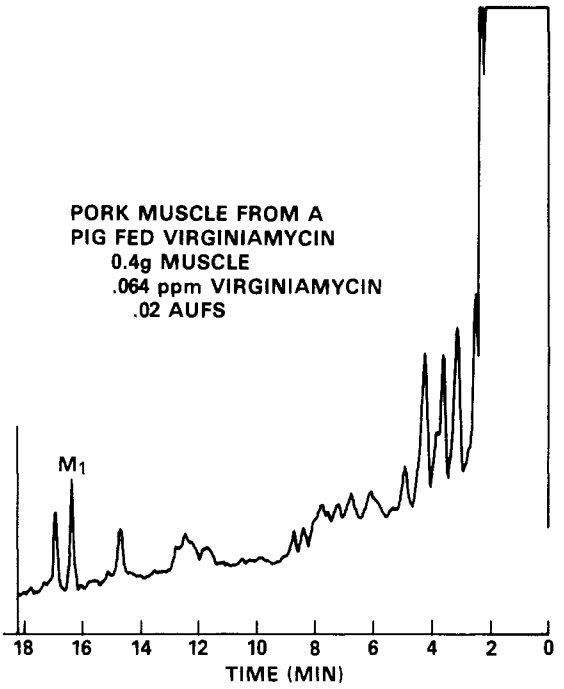

Figure 6. Pork muscle from animal fed 1000 ppm virginiamycin. Conditions as in Figure 4.

control pigs treated identically were also used.

The results are summarized in Table II. Samples from the control pigs showed little interference at the retention time of virginiamycin. Results with muscle from a control and a dosed pig are shown in Figures 5 and 6, respectively. Four 200-mL injections were used to increase sensitivity. As little as 0.01 ppm could be detected in all samples under these conditions. Results show low but readily detectable levels in both blood and tissues. The lower levels found in liver suggest that it is bound or metabolized in this tissue. The presence in blood had been inferred in earlier animal feeding studies since it was excreted in the urine (De Somer and Van Dijck, 1955). However, the bioassays were not sensitive enough to detect it directly. Since the feeding levels used of 10 times the highest recommended level (Leidahl, 1984) did not produce residues above the lowest tolerance limits of 0.1 ppm in muscle (Brown, 1985), it is unlikely that virginiamycin will be a residue problem. These low levels are unlikely to be detected by bioassays.

This method will enable regulatory agencies to determine whether virginiamycin is present in tissues containing unknown microbial inhibitors. It should also be adaptable to determination of virginiamycin in formulations and livestock feeds.

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Registry No. Virginiamycin M_1, 21411-53-0; virginiamycin, 11006-76-1; virginiamycin S, 9040-14-6.

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