Effect of Antibiotic Combination, Dosing Period, Dose Vehicle, and Assay Method on Salinomycin Residue Levels and Their Ionophoricity in Chicken Tissues

Gary P. Dimenna,* F. Scott Lyon, Frank M. Thompson, James A. Creegan, and George J. Wright

Studies were conducted to determine the effect of antibiotic combinations (lincomycin or bacitracin MD and roxarsone), length of dosing period, pretreatment with medicated feed, and dose vehicle on salinomycin residue levels in chicken tissues. Salinomycin was determined by high-performance liquid chromatography and thin-layer chromatography. Ionophoric activity was determined by a radiolabeled rubidium binding assay. Salinomycin residue levels were unaffected by treatment and assay method. Total residue levels were less than the established tolerance limits for muscle and skin/fat but approached the tolerance limit of 1.8 ppm for liver. Total drug residue levels in liver could be estimated by unchanged salinomycin level in skin/fat. Unchanged salinomycin did not account for all of the ionophoric activity present in liver. The affinity for rubidium binding by the salinomycin metabolites is approximately 20% of that of unchanged drug. Since liver metabolites have minimal ionophoric activity, the residues would have no toxicologic consequences.

Sodium salinomycin (Figure 1) is a polyether, carboxylic ionophore currently being marketed for its anticoccidial activity in chickens at a use level of 40–60 g/ton. Previous residue studies with unlabeled drug conducted at our facilities suggested that feeding salinomycin in combination with other antibiotics may affect the level of salinomycin in skin/fat, which was designated as the target tissue. The levels of salinomycin in skin/fat as determined by a thin-layer bioautographic method similar to that described by Dimenna et al. (1986b) were 0.039, 0.029, and 0.200 ppm, when salinomycin and roxarsone were fed in combination with bacitracin MD, zinc bacitracin, or virginiamycin, respectively. The levels of salinomycin in skin/fat as determined by a high-performance liquid chromatographic (HPLC) method described by Dimenna et al. (1986a) were 0.180, <0.100, and <0.100 ppm, when salinomycin and roxarsone were fed in combination with lincomycin, oxytetracycline, or chlortetracycline, respectively. Because of these results, residue studies were conducted to determine the effect of antibiotic combination and assay methodology on salinomycin residue levels in chicken tissues.

In the first study (hereafter designated study A) unchanged salinomycin levels were determined in chicken tissues by two independent assay methods: HPLC and radiometric thin-layer chromatography (TLC). Salinomycin (75 g/ton) was administered by itself or in combination with roxarsone plus lincomycin for 20 days in the feed, followed by oral administration by gavage of [^{14}C]salinomycin at 6.7 mg/kg per day (equivalent to 75 g/ton) in sodium bicarbonate for 5 days. The HPLC method of Dimenna et al. (1986a) was modified in order to lower the quantifiable limit of detection. During the course of study A, it was discovered that the liver total drug residue levels were at the tolerance limit of 1.8 ppm and were not affected by antibiotic combination. This was in contrast to an earlier residue study, in which chickens not pretreated with salinomycin in the feed prior to being dosed with [^{14}C]salinomycin for 5 days at 5.3 mg/kg per day (equivalent to 60 g/ton) had a total residue level in liver of 0.39 ppm. In response, an additional study (hereafter designated study B) was conducted to determine whether salinomycin residue levels were affected by pretreatment with salinomycin in the feed prior to dosing with [^{14}C]salinomycin, antibiotic combination, length of dosing period of [^{14}C]salinomycin, and dosing with [^{14}C]salinomycin in a feed slurry vs a sodium bicarbonate solution.

It was discovered that during the course of the salinomycin residue studies described here that mean total drug residue levels in chicken liver were approaching or were at the tolerance levels of 1.8 ppm. Toxicologic and pharmacologic activities of ionophores, such as salinomycin, are related to their ability to form electrically neutral zwitterionic complexes with cations, thus acting as vehicles for transporting ions across biological membranes and disturbing numerous intracellular functions (Pressman and Fahim, 1982; Reed, 1982). A ^{86}Rb radiolabeled binding assay, similar to the ^{22}Na radiolabeled binding assay for monensin described by Fahim and Pressman (1981), was developed for the determination of ionophoric activity in liver tissue extracts from chickens dosed with salinomycin. Unchanged salinomycin accounts for only 3% of the total salinomycin residue in chicken liver, and the data of Miyazaki et al. (1976) revealed that synthetic derivatives of salinomycin have varying degrees of ionophoric and antimicrobial activity depending on which functional group of salinomycin was altered. It would follow then that the salinomycin residue from treated chickens may have ionophoric activity both quantitatively and/or qualitatively different from that of the parent drug. Therefore, the ionophoric activity of the total salinomycin residue in liver was assessed, because salinomycin expresses its pharmacologic and toxic activity via its ionophoricity.

MATERIALS AND METHODS

Salinomycin Standards. The standard substances were sodium salinomycin (potency of 926 μg/mg) and ^{14}C-labeled sodium salinomycin ([^{14}C]salinomycin). The radiopurity of [^{14}C]salinomycin was >98% by TLC in several systems. The internal standard (IS) was sodium narasin (potency 934 μg/mg), obtained from Eli Lilly and Co., Indianapolis, IN.

Materials and Equipment for Sample Preparation. The following materials and equipment were used for sample preparation: Polytron Model PT-35 tissue homogenizer with a Model PT-20ST probe generator (Brinkmann Instruments, Inc., Westbury, NY); Tri-Carb Model 306 oxidizer, Models 3385 and 4530 liquid scintillation spectrometers, Carbo-Sorb, Permafluor

Department of Drug Metabolism, A. H. Robins Company, Richmond, Virginia 23261-6609.

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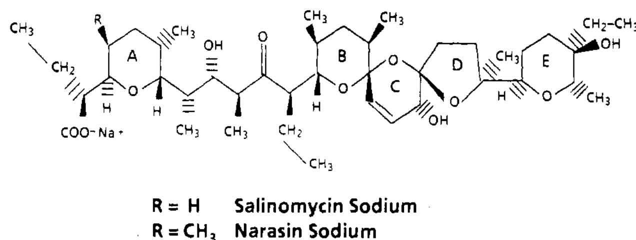

Figure 1. Structure of sodium salinomycin (R = H) and sodium narasin (R = CH_3).

V, and Insta-Gel (Packard Instrument Co., Downers Grove, IL); IEC Model V centrifuge (Boston, MA); 0.2- and 0.5-g silica gel Bond-Elut columns, preparative-grade Sepralyte silica gel, and Vac-Elut vacuum manifold (Analytichem International, Harbor City, CA); flash chromatography column (J. T. Baker Chemical Co., Phillipsburg, NJ); silica Sep-Pak cartridge (Waters Associates, Milford, MA); 20 × 20 cm, 250-μm-thickness, LK5F linear-K, silica gel TLC plates (Whatman, Milford, MA); Type SB-5; single-coated, blue-sensitive X-ray film (Eastman Kodak Co., Rochester, NY); a 24-position nitrogen evaporator (Organomation, South Berlin, MA); Gilson pipettors (Rainin Instruments); Buchi Rotavapor-R rotary evaporator (Brinkmann); and polypropylene 1.5-mL microcentrifuge tubes (VWR, Baltimore, MD).

Reagents and Solvents. Metal chlorides and organic buffers were purchased from Sigma Chemical Co. (St. Louis, MO). Rubidium-86 chloride (^{86}Rb, specific activity 0.25 mCi/μmol) was purchased from Amersham Corp. (Arlington Heights, IL). All of the solvents were either nanograde or HPLC grade and were purchased from J. T. Baker or Burdick and Jackson (Muskegon, MI).

Dosing Solution for Study A. The dosing solution was prepared by dissolving 0.2632 g of sodium salinomycin in 25 mL of a solution of [^{14}C]salinomycin (0.3385 g) in ethanol. The solvent was evaporated under a stream of nitrogen. Salinomycin was dissolved in 432 mL of 0.01 M sodium bicarbonate by stirring for 2 h at room temperature. The final concentration of salinomycin in the dosing solution was 1.38 mg/mL with a specific activity of 13.1 μCi/mg.

Dosing Solutions for Study B. The dosing solutions were prepared by dissolving 0.7766 g of sodium salinomycin in 19 mL of a solution of [^{14}C]salinomycin (0.4540 g) in ethanol. The solvent was evaporated under a stream of nitrogen. Salinomycin was dissolved in 400 mL of 0.01 M sodium bicarbonate by stirring for 4 h at room temperature. To 138 mL of this solution was added 35 g of milled, poultry starter mash and the mixture homogenized with a Polytron homogenizer. The remainder of the [^{14}C]salinomycin solution in sodium bicarbonate (262 mL) was diluted with 72 mL of 0.01 M sodium bicarbonate. The concentration of salinomycin was 1.77 mg/mL for the feed slurry and 1.66 mg/mL for the sodium bicarbonate solution. The specific activity was 10.0 μCi/mg for the feed slurry and 9.7 μCi/mg for sodium bicarbonate solution.

The radioisotope purity was >98% by TLC in several systems. [^{14}C]Salinomycin was stable throughout the dosing period in the dosing solutions. The dosing solutions were stored at 4 °C and warmed to room temperature prior to dosing.

Dosing Chickens. One-day-old (day 1 of the study) Hubbard × Hubbard chicks were fed unmedicated starter mash through day 11. On day 12 the chickens were randomized into the nine treated groups described in Table I. Control groups, chickens fed unmedicated feed throughout the study, were also included. The number of chickens in groups I–VIII was culled to 6 on day 29 and in group IX was culled to 10 on day 29. Artificial light was maintained 24 h/day throughout the studies. On day 32 the chickens were weighed and switched to salinomycin-free feed, and dosing with [^{14}C]salinomycin commenced. The dose for the individual chickens was based on the estimated weight of that chicken midway through the dosing period. The estimated weights at the midpoints of the 5- and 2-day dosing periods were 110% and 102%, respectively, of their weights on day 32. Each chicken was dosed orally by gavage at 12-h intervals with [^{14}C]salinomycin at 3.37 mg/kg per dose. This dose was estimated to be equivalent to 75 g/ton of feed. Water was offered ad libitum throughout

Table I. Summary of Medicated Diets and Dosing Regimens for the Treated Groups

treatment group[b]	diet[a] at days		
	12–31	32–33	32–36
I	S		U/[^{14}C]S[c]
II	S/R/L		R/L/[^{14}C]S[c]
III	U	U/[^{14}C]S[c]	
IV	U		U/[^{14}C]S[c]
V	S	U/[^{14}C]S[c]	
VI	S		U/[^{14}C]S[c]
VII	S		U/[^{14}C]S[d]
VIII	S/R/BMD		R/BMD/[^{14}C]S[c]
IX	S		U/[^{14}C]S[c]

[a] Abbreviations are as follows: U = unmedicated, S = salinomycin at 75 g/ton, R = roxarsone at 45 g/ton, L = lincomycin at 4 g/ton, BMD = bacitracin MD at 200 g/ton. [b] Treatment groups I and II are from study A, and treatment groups III–IX are from study B. Both studies had control groups that were not dosed with antibiotic. [c] [^{14}C]S = [^{14}C]salinomycin in a sodium bicarbonate solution given by gavage. [d] [^{14}C]S = [^{14}C]salinomycin in a feed suspension in sodium bicarbonate given by gavage.

the study. Feed was offered ad libitum throughout the study until administration of the last dose of [^{14}C]salinomycin; afterward, feed was removed, and 6 h later (zero-time withdrawal) the chickens were killed.

Tissue Homogenization. A portion of the skin (feathers removed) from the breast and legs with accompanying subcutaneous fat, a portion of muscle from the breast and legs, and the entire liver were removed, weighed, and immediately frozen until analysis. The tissues were thawed, cut into small pieces, weighed, and homogenized in water (skin/fat/water, 20/80, w/v; liver/water and muscle/water, 20/46, w/v).

Total Drug Residue Analysis. Total drug residue levels were determined by combusting 1.0 mL of the homogenate and counting the $^{14}CO_2$ in Permafluor V by liquid scintillation spectrometry. The tissue homogenates were stored frozen.

Tissue Preparation for Analysis by HPLC. Duplicate 10-mL aliquots of liver homogenate (equivalent to 3 g of tissue) or duplicate 5-mL aliquots of skin/fat homogenate (equivalent to 1 g of tissue) were pipetted into centrifuge tubes and then spiked with 100 μL of an IS solution in methanol. For the liver standard curves, 10-mL aliquots of control tissue homogenate were spiked with 100 μL of salinomycin and IS standard solutions in methanol producing a standard curve with salinomycin concentrations of 0, 0.005, 0.01, 0.02, 0.04, 0.10, and 0.20 ppm and an IS concentration of 0.10 ppm. For the skin/fat standard curves, 5-mL aliquots of control tissue homogenate were spiked with 100 μL of salinomycin and IS standard solutions in methanol producing a standard curve with salinomycin concentrations of 0, 0.02, 0.04, 0.06, 0.08, 0.10, and 0.20 ppm and an IS concentration of 0.20 ppm.

Unchanged salinomycin was extracted from the homogenate by being shaken with isooctane (30 mL for liver and 36 mL for skin/fat) on a reciprocal shaker for 15 min. The samples were then centrifuged at room temperature for 10 min at 2000 rpm, and the isooctane was transferred to a 0.5-g silica column. The column was washed with 20 mL of methylene chloride and then 10 mL of methylene chloride/methanol (985/15, v/v). Salinomycin and IS were eluted from the column with 6 mL of methylene chloride/methanol (9/1, v/v). The eluate was evaporated under a stream of nitrogen, reconstituted in 1 mL of methylene chloride, and transferred to a 16 × 100 mm culture tube containing approximately 5 mg of pyridinium dichromate. Pyridinium dichromate was synthesized according to the method of Corey and Schmidt (1979). Pyridinium dichromate oxidizes the allylic hydroxyl group, forming an α,β-unsaturated ketone with a λ_{max} at 225 nm. The tubes were shaken for 8 min at room temperature, which is sufficient time for the reaction to go to completion. The reaction was stopped by adding 1 mL of 5% sodium bicarbonate, vortexing, and then adding 3 mL of methylene chloride and vortexing. The aqueous layer was removed, and the methylene chloride layer was washed an additional four times with 1-mL portions of 5% sodium bicarbonate and once with 1 mL of water. The methylene chloride layer was dried over anhydrous, powdered

sodium sulfate and transferred to a 0.2-g silica column. The column was washed with 6 mL of methylene chloride, and oxidized salinomycin and IS were eluted with 3 mL of methylene chloride/methanol (9/1, v/v). The eluate was evaporated to dryness under a stream of nitrogen, reconstituted in 110 μL of acetonitrile, and transferred to an autosampler vial.

HPLC System. Salinomycin and IS were chromatographed according to the HPLC system of Dimenna et al. (1986a). The HPLC system utilized column switching and UV detection at 225 nm. An Hitachi autosampler (Model 655-A40; E. M. Science, Cherry Hill, NJ) was used with an injection volume of 100 μL.

An equation for the least-squares regression line was calculated by comparing the peak height ratio of salinomycin to IS with known concentrations of salinomycin. This equation was used to determine the concentration of salinomycin in the unknown samples.

Tissue Preparation for Analysis by TLC. Duplicate 10-mL aliquots of liver homogenate (equivalent to 3 g of tissue) or duplicate 15-mL aliquots of skin/fat homogenate (equivalent to 3 g of tissue) were pipetted into 100-mL centrifuge tubes. For the standard curves, aliquots of control tissue homogenate were spiked with 100 μL of [^{14}C]salinomycin standard solutions in methanol, producing a standard curve with salinomycin concentrations of 0, 0.005, 0.010, 0.030, 0.075, 0.125, and 0.200 ppm.

Unchanged salinomycin was extracted from the homogenate by being shaken with 45 mL of isooctane. The samples were centrifuged for 15 min at 2000 rpm. The supernatant was passed through a silica Sep-Pak cartridge. The cartridge was washed with 10 mL of methylene chloride/methanol (985/15, v/v) and eluted with 10 mL of methylene chloride/methanol (9/1, v/v). The extract was evaporated to dryness under a stream of nitrogen, reconstituted in methanol, and transferred to autosampler vials. The extract was evaporated to dryness under a stream of nitrogen and reconstituted in 60 μL of toluene.

TLC System. Fifty microliters of each extract in toluene and a reference solution of [^{14}C]salinomycin were applied to the TLC plate. This spotting scheme allowed for nine samples and one reference spot per plate. The plates were developed to approximately 15 cm from the origin in 100 mL of 25% trimethylamine in methanol/benzene/*n*-heptane (25/90/90, v/v/v), air-dried, and then exposed to X-ray film for 2–3 days. The [^{14}C]salinomycin zones were located. The silica was scraped directly into scintillation vials, suspended in 8 mL of water, and counted in Insta-Gel.

An equation for the least-squares regression line was calculated by comparing the disintegration per minute values with known concentrations of salinomycin. This equation was used to determine the concentration of salinomycin in unknown samples.

Preparation of Liver Tissue for Ionophore Analysis. Quadruplicate 3.4-mL aliquots of liver homogenates (equivalent to 1 g of tissue) were pipetted into centrifuge tubes. For the standards, quadruplicate 3.4-mL aliquots of control tissue (study control) homogenate were spiked with 100 μL of [^{14}C]salinomycin standard solutions in methanol, resulting in salinomycin concentrations of 0, 0.51, 1.05, and 2.09 ppm for the first study and 0, 0.195, 0.388, 0.569, 0.782, and 0.927 ppm for the second study.

Extractable drug residue was extracted from the homogenate by being shaken with 20 mL of ethyl acetate for 15 min. The samples were then centrifuged for 20 min at 3000 rpm. The ethyl acetate was transferred to glass vials, evaporated under a stream of nitrogen, and reconstituted in 2 mL of methylene chloride. The extract was transferred to a 0.5-g silica column, and the column was washed with 6 mL of methylene chloride and then 10 mL of methylene chloride/methanol (985/15, v/v). Extractable drug residue was eluted with 10 mL of chloroform/methanol/concentrated ammonium hydroxide (900/100/10, v/v/v), dried under a stream of nitrogen, and reconstituted in 2 mL of toluene/butanol (7/3, v/v). A 0.1-mL aliquot of the extract was removed for determination of recovery of ^{14}C by counting in Insta-Gel.

For determination of ionophoric activity in the tissue extract, 1 mL of an aqueous buffer solution (27 mL of 50 mM DMG, pH to 11.0 with 1 N tetramethylammonium hydroxide (Me_4NOH), 3 mL of 0.2 mM RbCl in water, and 0.3 mL of ^{86}RbCl (0.1 mCi/mL of water)) was added to the extract in toluene/butanol, vortexed twice for 30 s each, and centrifuged for 10 min at 1000 rpm. The amount of ^{86}Rb bound by the tissue extract was determined by counting a portion of the organic phase in Insta-Gel. The window settings on the scintillation counter were adjusted to subtract out the β-emissions resulting from ^{14}C, in order to count ^{86}Rb.

Preparation of Liver Tissue for pH Effect of Ionophore Activity. The male and female liver homogenates from group IX of study B were pooled and then assayed for the effect of pH and cation competition on ^{86}Rb binding. Control liver homogenate and the male and female liver homogenates (53 mL; equivalent to 15.9 g of tissue) were added to 250-mL, disposable, screw-cap centrifuge tubes. The control homogenate was spiked with 500 μL of [^{14}C]salinomycin standard solutions, resulting in salinomycin concentrations of 0, 0.10, 0.21, 0.32, 0.45, 1.09, and 1.99 ppm.

Extractable drug residue was extracted twice from the homogenate by being shaken with 150 mL of ethyl acetate for 15 min. The extracts were taken to dryness in a rotary evaporator. The extract was reconstituted in 50 mL of methylene chloride and then added to a 15 g silica gel column. The column was washed with 200 mL of methylene chloride, followed by 300 mL of methylene chloride/methanol (985/15, v/v). The drug residue was eluted with 500 mL of chloroform/methanol/concentrated ammonium hydroxide (900/100/10, v/v/v), taken to dryness in a rotary evaporator, reconstituted in 15 mL of chloroform, and transferred to centrifuge tubes. The drug residue was converted to the free-acid form by being shaken with 2 mL of 0.01 N acetic acid and then filtered through phase-separating paper (Whatman 1PS). The solvent was evaporated under a stream of nitrogen and the extract reconstituted in 25 mL of toluene/butanol (7/3, v/v). A 0.1-mL portion of the extract was removed for determination of recovery of ^{14}C by counting in Insta-Gel.

The extracts were then assayed for ionophoric activity by adding 0.8-mL aliquots of the extract to 1.5-mL microcentrifuge tubes containing 0.2 mL of buffer, 0.1 mL of 100 μM RbCl in water, and 0.1 mL of ^{86}RbCl (10 μCi/mL of water). Various buffers, depending on their pK_a value, were used in the assay milieu. Stock solutions of buffer at 50 mM in deionized, distilled water were prepared, and the pH was adjusted with 1 N Me_4NOH. The buffers and their final pH values were as follows: MES at pH 5.00 and 6.01; HEPES at pH 7.00; TAPS at pH 8.01 and 9.03; DMG at pH 9.52, 10.02, 10.51, 11.03, and 11.70. Each pH point was run in triplicate. The microcentrifuge tubes were vortexed for 1 min and then centrifuged at 2500 rpm for 5 min. The amount of ^{86}Rb bound by the tissue extract was determined by counting of an aliquot of the organic phase in Insta-Gel.

Preparation of Liver Tissue for Cation Effect of Ionophoric Activity. To determine the effect of competing cations on the binding of ^{86}Rb at pH 9.50 and 11.04, the liver homogenates were extracted and purified as described in the pH profile experiment with minor exceptions. Thirty-three milliliters (equivalent to 9.9 g of tissue) of homogenate was extracted with ethyl acetate, and the final purified extract was reconstituted in 15 mL of toluene/butanol (7/3, v/v). Control liver homogenate was spiked with [^{14}C]salinomycin at 0, 0.337, 1.285, and 1.943 ppm and at 0, 0.116, 0.342, and 1.902 ppm for assay at pH 9.50 and 11.04, respectively. The extracts were then assayed for ionophoric activity by adding 0.8-mL aliquots of the tissues extract to microcentrifuge tubes containing 0.1 mL of buffer (50 mM DMG at pH 9.50 or 11.04), 0.1 mL of ^{86}RbCl (10 μCi/mL of buffer), 0.1 mL of 100 μM RbCl in water, and 0.1 mL of one of the following: water, 100 μM RbCl, 100 μM KCl, 100 μM NaCl, 50 μM $CaCl_2$, or 50 μM $MgCl_2$. The assay was completed as described in the previous experiment.

RESULTS AND DISCUSSION

HPLC Method. The quantifiable limits of detection were 0.020 ppm for skin/fat and 0.005 ppm for liver. The method gave a linear response with concentrations of salinomycin from 0.020 to at least 0.200 ppm in skin/fat and from 0.005 to at least 0.200 ppm in liver. We have found that isooctane is as effective as methanol or methyl *tert*-butyl ether in extracting salinomycin from tissues of animals dosed with salinomycin. Extraction of tissue homogenates with isooctane rather than methanol yields a much purer extract, which allowed injection of the entire extract onto the HPLC column and lowered the detection limits over the HPLC method previously developed in our

Table II. HPLC and TLC Determination of Salinomycin in Spiked Tissue Homogenates

HPLC				TLC			
added, ppm	tissue	found ppm[a]	found % CV	added, ppm	tissue	found ppm[b]	found % CV
0	skin/fat	BQL[c]		0	skin/fat	BQL[d]	
0.030	skin/fat	0.029	9.9	0.008	skin/fat	0.008	10.5
0.050	skin/fat	0.050	4.1	0.009	skin/fat	0.008	17.5
0.075	skin/fat	0.074	1.0	0.021	skin/fat	0.022	7.4
0.090	skin/fat	0.090	3.2	0.022	skin/fat	0.021	15.9
0.150	skin/fat	0.159	4.4	0.035	skin/fat	0.035	12.8
0	liver	BQL[d]		0.062	skin/fat	0.061	13.2
0.006	liver	0.007	17.6	0.067	skin/fat	0.070	14.4
0.018	liver	0.017	6.5	0.123	skin/fat	0.125	6.1
0.036	liver	0.034	4.5	0.164	skin/fat	0.168	9.5
0.054	liver	0.050	3.3	0.166	skin/fat	0.172	15.8
0.090	liver	0.081	2.4	0	liver	BQL[d]	
0.180	liver	0.157	1.4	0.007	liver	0.006	25.2
				0.018	liver	0.016	16.4
				0.052	liver	0.049	6.8
				0.103	liver	0.098	6.7
				0.139	liver	0.135	10.0

[a] Each determination is a mean of five homogenate samples spiked in duplicate. [b] Each determination is a mean of six homogenate samples spiked in duplicate for skin/fat and eight homogenate samples spiked in duplicate for liver. [c] BQL = below quantifiable limit (<0.020 ppm for skin/fat). [d] BQL is <0.005 ppm.

laboratory (Dimenna et al., 1986a).

Typical chromatograms of extracts of spiked control skin/fat and liver homogenates are shown in Figures 2 and 3, respectively. Base-line separation was achieved between salinomycin and the IS. Roxarsone, bacitracin, lincomycin, virginiamycin, chlortetracycline, and oxytetracycline did not produce any peaks or interfere with the determination of salinomycin. This method is specific for salinomycin and narasin, since it requires formation of a chromophore by oxidation of the allylic hydroxyl group forming an α,β-unsaturated ketone. Therefore, monensin and lasalocid are not detected by this HPLC method.

Accuracy and precision of the HPLC method were determined by assaying tissue homogenates spiked with various concentrations of salinomycin (Table II). The concentrations of salinomycin in the samples were unknown to the analyst at the time the samples were assayed. The results demonstrate that the method has a high degree of accuracy and precision.

TLC Method. The limit of detection was 0.005 ppm for both skin/fat and liver. The TLC method gave a linear response for concentrations of salinomycin from 0.005 to at least 0.200 ppm in skin/fat and liver. The accuracy and precision of the TLC method were determined in the same manner as for the HPLC method. The results in Table II show that the TLC method is as accurate as the HPLC method but more variable.

Stability. Liver and skin/fat homogenates were spiked with salinomycin at 0.100 ppm, immediately frozen, and then assayed for salinomycin by the HPLC method 3 and 9 weeks later. All of the analyses of the unknown samples from the residue studies were completed within 9 weeks of collecting the samples. The results in Table III show that salinomycin was stable in spiked samples stored under conditions similar to those of the unknown samples.

Tissue Residue Levels. Total drug residue and unchanged salinomycin data for the control groups from both studies are not shown, since all values obtained were below the quantifiable limits of the assay methods. Total drug residue levels were determined in muscle for study B only. The total drug residue levels in muscle ranged from 0.025 to 0.037 ppm and were not affected by any of the treatments. These levels are well below the tolerance limit of 0.6 ppm for chicken muscle.

Total residue and unchanged salinomycin levels obtained in skin/fat are presented in Table IV. The tolerance limit for total residue in skin/fat is 1.2 ppm, and the levels obtained in both studies are appreciably lower than the tolerance limit. Unchanged salinomycin is the major component of the total residue in skin/fat, accounting for approximately 70% of the residue. Therefore, unchanged salinomycin in skin/fat is a suitable marker substance for total drug residue. Both assay methods gave similar values for salinomycin, which supports the acceptability of the HPLC method.

Steady-state levels of total drug residue and unchanged salinomycin in skin/fat are obtained after 5 days of dosing with [^{14}C]salinomycin. Residue levels were not affected by antibiotic combination and dose vehicle. The apparent differences between the treatment means are not significant, due to the large variation between samples.

The liver residue values obtained from the studies are presented in Table V. The tolerance limit for total residue in liver is 1.8 ppm. The total residue levels obtained in

Table III. Stability of Salinomycin in Frozen Tissue Homogenates[a]

salinomycin added, ppm	tissue	length of storage, weeks	salinomycin found, ppm
0.100	skin/fat	3	0.105 ± 0.0060[b]
0.100	skin/fat	9	0.101 ± 0.0037
0.100	liver	3	0.111 ± 0.0013
0.100	liver	9	0.096 ± 0.0016

[a] Tissue homogenates were spiked with salinomycin at 0.100 ppm, immediately frozen, and then assayed for salinomycin by HPLC after 3 and 9 weeks of storage. [b] Mean ± SD.

Table IV. Total Drug Residue Levels and Unchanged Salinomycin Levels by HPLC and TLC in Skin/Fat[a]

group	total drug residue, ppm	salinomycin by HPLC, ppm	salinomycin by TLC, ppm
I	0.237 ± 0.024	0.150 ± 0.035	0.128 ± 0.038
II	0.319 ± 0.238	0.190 ± 0.157	0.156 ± 0.101
III	0.137 ± 0.104	0.079 ± 0.049	0.086 ± 0.041
IV	0.186 ± 0.051	0.131 ± 0.051	0.147 ± 0.041
V	0.122 ± 0.043	0.092 ± 0.050	0.105 ± 0.046
VI	0.193 ± 0.087	0.129 ± 0.049	0.133 ± 0.047
VII	0.217 ± 0.176	0.176 ± 0.155	0.188 ± 0.164
VIII	0.176 ± 0.029	0.145 ± 0.048	0.134 ± 0.042

[a] Values are means ± SD.

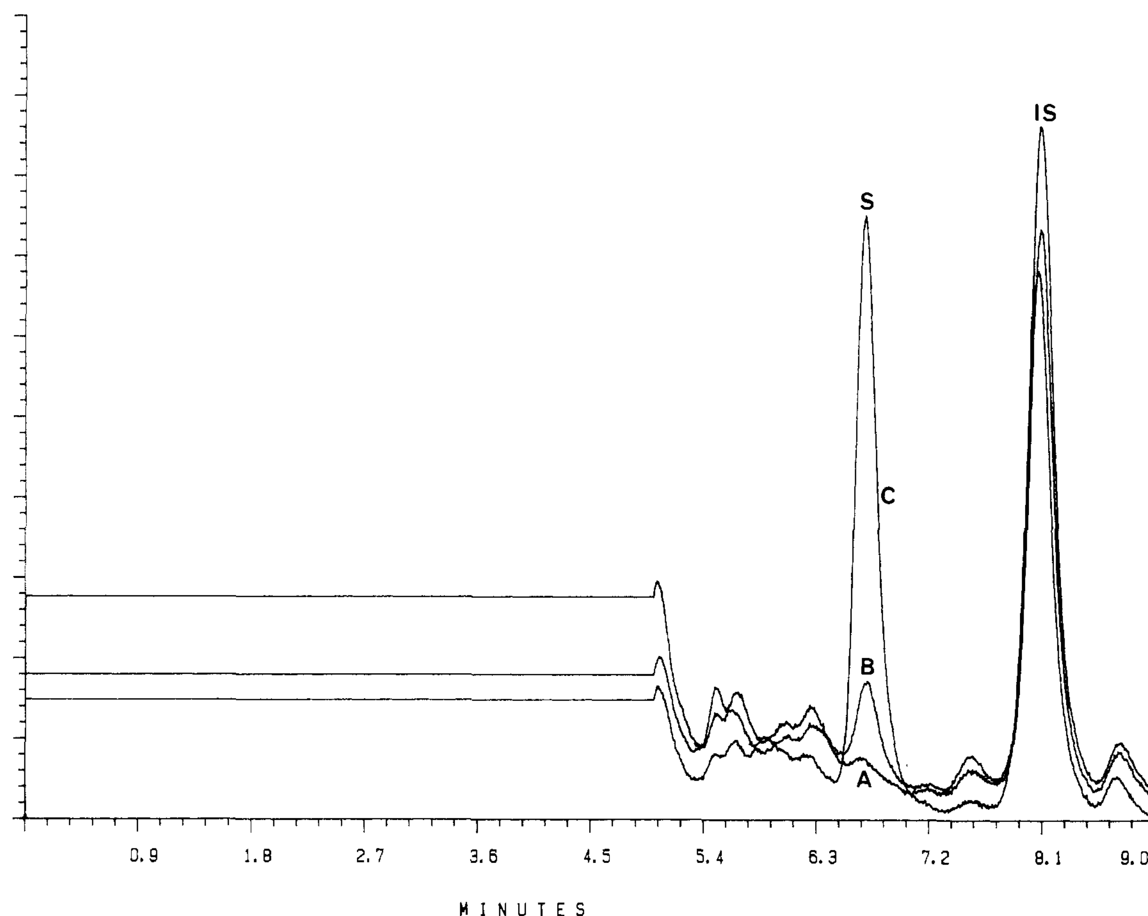

Figure 2. Multiple plot chromatograms of control skin/fat tissue spiked with (A) 0 ppm salinomycin, (B) 0.02 ppm salinomycin, and (C) 0.20 ppm salinomycin (S = salinomycin, IS = internal standard at 0.2 ppm).

Table V. Total Drug Residue Levels and Unchanged Salinomycin Levels by HPLC and TLC in Liver[a]

group	total drug residue, ppm	salinomycin by HPLC, ppm	salinomycin by TLC, ppm
I	1.941 ± 0.345	0.053 ± 0.031	0.058 ± 0.037
II	1.851 ± 0.906	0.064 ± 0.045	0.070 ± 0.055
III	1.174 ± 0.618	0.032 ± 0.025	0.032 ± 0.023
IV	1.616 ± 1.182	0.042 ± 0.024	0.043 ± 0.022
V	1.182 ± 0.872	0.026 ± 0.012	0.026 ± 0.014
VI	1.192 ± 0.623	0.045 ± 0.028	0.042 ± 0.022
VII	1.470 ± 0.830	0.062 ± 0.056	0.064 ± 0.058
VIII	2.142 ± 0.699	0.085 ± 0.021	0.077 ± 0.017

[a] Values are means ± SD.

study A are at the tolerance level but were not affected by feeding salinomycin in combination with roxarsone and lincomycin. The total residue levels obtained with the salinomycin/roxarsone/bacitracin MD combination in study B were 19% higher than tolerance limit.

Steady-state levels of total drug residue and unchanged salinomycin are achieved by dosing with [^{14}C]salinomycin for 5 days. The TLC method determines the concentration of unchanged [^{14}C]salinomycin on the basis of the specific activity of the dose given, while the HPLC method determines the concentration of total unchanged salinomycin. If significant accumulation of unlabeled salinomycin occurred in skin/fat and liver from feeding salinomycin prior to dosing with [^{14}C]salinomycin, then the HPLC method would give a higher value for unchanged drug than would the TLC method. This did not occur, suggesting that salinomycin is eliminated from chicken tissues rapidly enough to prevent accumulation of drug. Therefore, inclusion of salinomycin in the diet prior to dosing with [^{14}C]salinomycin would not account for the increased residue levels obtained with these studies as opposed to the 0.39 ppm total residue level obtained in liver and 0.06 ppm total residue level obtained in skin/fat in an earlier residue study conducted at our facilities, in which non-pretreated chickens were dosed with [^{14}C]salinomycin by gavage for 5 days at a level equivalent to 60 g/ton.

In study B the addition of roxarsone and bacitracin MD to the diet had little effect on residue levels. However, the residue levels obtained in this study with the salinomycin/roxarsone/bacitracin MD combination were greater than those obtained in the previously mentioned residue study conducted at our facilities. In that study the chickens were dosed with unlabeled salinomycin in the feed. It would follow that the increased residue levels obtained here would be attributed to the chickens receiving half of their daily dose of [^{14}C]salinomycin at once by gavage as opposed to inclusion of the dose in the feed. However, the residue levels obtained with the salinomycin/roxarsone/lincomycin combination group are consistent with an earlier salinomycin/roxarsone/lincomycin combination study, in which chickens were dosed with unlabeled salinomycin in the feed. The results reported here show that residue levels are not affected by dosing with [^{14}C]salinomycin in a feed slurry or in a sodium bicarbonate solution.

The highest residue levels were found in liver. Unfortunately, unchanged salinomycin is not a suitable marker for total drug residue in liver, since it accounted for only 3% of the total drug residue. However, the ratio of total drug residue level in liver to unchanged salinomycin level in skin/fat was fairly constant between treatment groups (Table VI). Therefore, the concentration of unchanged

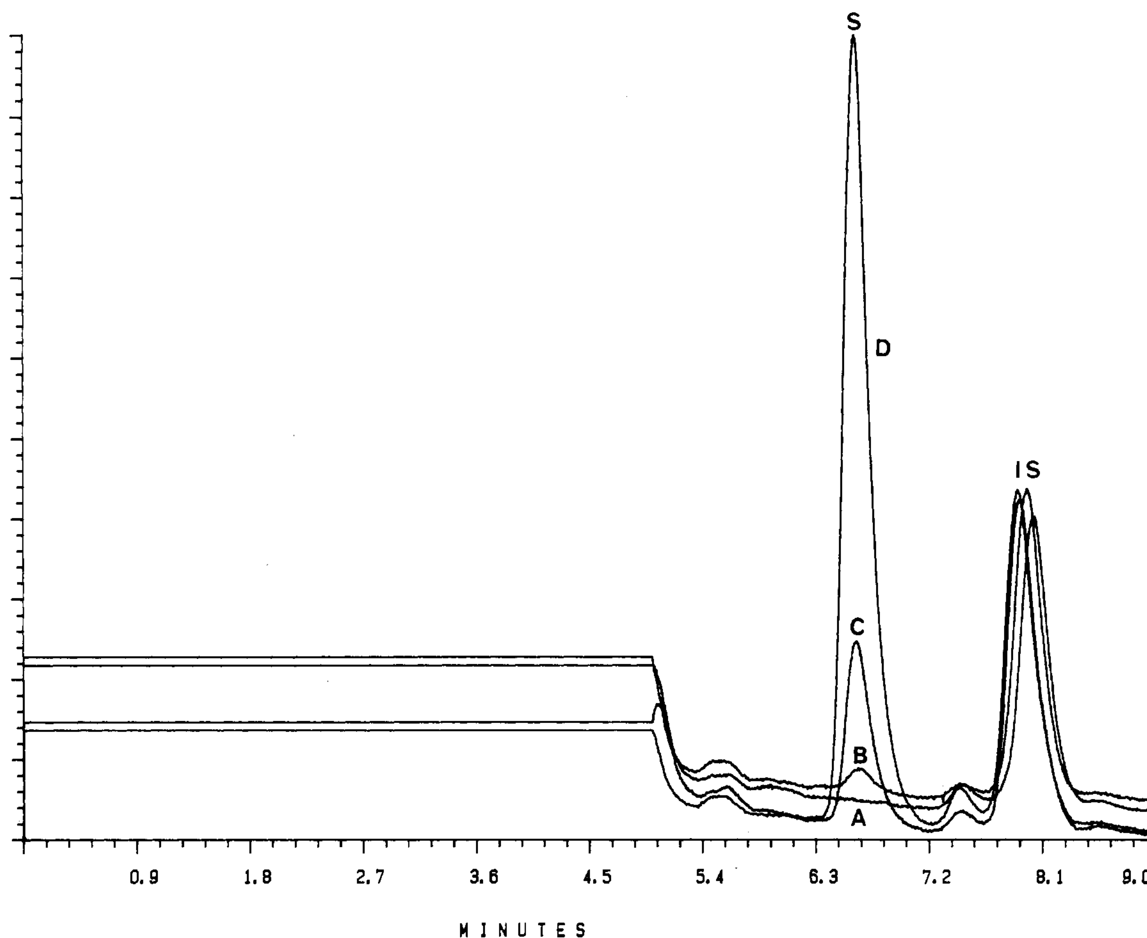

Figure 3. Multiple plot chromatograms of control liver tissue spiked with (A) 0 ppm salinomycin, (B) 0.005 ppm salinomycin, (C) 0.04 ppm salinomycin, and (D) 0.2 ppm salinomycin (S = salinomycin, IS = internal standard at 0.1 ppm).

Table VI. Ratio of Total Drug Residue Level in Liver to Unchanged Salinomycin Level in Skin/Fat

group	total residue in liver, ppm	unchanged salinomycin in skin/fat,[a] ppm	ratio
I	1.941	0.150	12.9
II	1.851	0.190	9.7
III	1.174	0.079	14.9
IV	1.616	0.131	12.3
V	1.182	0.092	12.8
VI	1.192	0.129	9.2
VII	1.470	0.176	8.4
VIII	2.142	0.145	14.8
mean			11.9
% CV			21.0

[a] Determined by HPLC.

Table VII. Cation Displacement of ^{86}Rb from Salinomycin-^{86}Rb Complex[a]

displacing cation	^{86}Rb bound, μmol	^{86}Rb remaining in complex, %
none	1.666	83.3
Rb^+	0.922	46.1
Na^+	1.404	70.2
K^+	0.725	36.3
Mg^{2+}	1.685	84.3
Ca^{2+}	1.661	83.1

[a] A 2-μmol portion of salinomycin-free acid in 2 mL of toluene/butanol (7/3) was equilibrated by shaking with 2 μmol of ^{86}RbCl in 1 mL of DMG/Me_4NOH buffer (40 mM; pH 11.3). Displacement of ^{86}Rb from the ionophore complex was determined by the addition of unlabeled cations (2 μmol of monovalent or 1 μmol of divalent metal chloride).

salinomycin in skin/fat would be an acceptable predictor of total drug residue levels in liver.

Ionophoric Activity. Since the levels of total salinomycin residue in liver were at the tolerance limit of 1.8 ppm for chicken liver, it was of importance to assess the biological significance of these residue levels by determining the ionophoric activity of the liver extracts. An assay was developed to measure the ionophoric activity of a liver extract. A purified liver extract in toluene/butanol was mixed with a buffered aqueous solution at pH 11 containing ^{86}Rb, and ^{86}Rb that partitions into the organic phase by being bound to salinomycin is taken as a measurement of ionophoric activity. Cation binding by salinomycin is pH dependent. Maximal binding occurs at pH 11, which is consistent with other ionophores, such as ionomycin (Liu and Hermann, 1978). Cation binding by salinomycin is selective, and salinomycin exhibits a preference for binding monovalent cations over that of divalent cations. Salinomycin selectively binds cations in the sequence $K^+ > Rb^+ > Na^+ > Ca^{2+}, Mg^{2+}$ (Table VII). These results are consistent with those of Mitani et al. (1975) and Miyazaki et al. (1976). For the measurement of ionophoric activity ^{86}Rb was used rather than ^{22}Na, since salinomycin exhibited a greater affinity for Rb^+ than Na^+.

The ^{86}Rb binding values obtained from the liver samples from the salinomycin residue studies are presented in Table VIII. Control liver homogenates from the studies were pooled, spiked with [^{14}C]salinomycin, and run concurrently with the treated samples. Recovery of total ^{14}C residue in the tissue extract was determined just prior to ^{86}Rb addition to the tissue extract. The mean recovery of ^{14}C in toluene/butanol for the [^{14}C]salinomycin stand-

Table VIII. Recovery of Total Drug Residue and Rubidium Binding in Liver from Chickens Dosed with Salinomycin and Control Liver Spiked with Salinomycin

treatment group[a]	chicken no.	sex	total residue,[b] ppm	% total ^{14}C recovered[c]	unchanged salinomycin,[d] ppm	^{86}Rb bound,[e] pmol	[^{14}C]salinomycin std, ppm	% ^{14}C recovered	^{86}Rb bound, pmol
I	2506	F	2.030	66.5	0.070	99.4 ± 11.98	0.000		27.1 ± 1.83
I	2507	F	2.155	70.4	0.069	116.6 ± 10.78	0.510	90.0	191.7 ± 11.01
I	2508	F	2.313	80.2	0.098	171.6 ± 10.97	1.050	90.7	365.8 ± 18.22
I	4507	M	1.310	64.0	0.030	74.4 ± 5.45	2.090	89.7	726.8 ± 15.57
I	4508	M	1.965	70.7	0.022	87.7 ± 7.65			
I	4510	M	1.873	68.2	0.026	88.0 ± 9.59			
II	2512	F	1.292	63.2	0.031	57.3 ± 7.22			
II	2514	F	1.323	69.6	0.034	74.1 ± 5.61			
II	2515	F	1.585	68.1	0.059	89.7 ± 3.87			
II	4514	M	3.451	76.0	0.141	213.8 ± 12.13			
II	4515	M	1.605	69.1	0.054	95.1 ± 2.41			
	2504[f]	F	0.000		0.000	34.2 ± 3.26			
	4501[f]	M	0.000		0.000	30.2 ± 4.72			
IX	9842	F	2.765	80.7	0.091	238.6 ± 15.85	0.000		27.2 ± 3.97
IX	9844	F	1.218	74.7	0.090	175.7 ± 1.28	0.195	109.0	111.9 ± 3.25
IX	9847	F	3.359	73.3	0.048	162.1 ± 9.87	0.388	93.6	173.5 ± 6.66
IX	9849	F	1.236	68.6	0.052	142.8 ± 10.70	0.569	93.6	243.6 ± 23.02
IX	9856	F	1.311	69.7	0.048	88.6 ± 15.76	0.782	91.2	326.4 ± 18.73
IX	3942	M	1.807	74.6	0.036	113.2 ± 3.32	0.927	95.4	397.5 ± 42.09
IX	3945	M	0.986	58.8	0.020	99.7 ± 14.17			
IX	3948	M	0.729	62.4	0.010	96.6 ± 19.93			
IX	3950	M	0.831	56.0	0.015	86.1 ± 3.56			
IX	3955	M	1.131	64.8	0.009	99.4 ± 13.89			

[a] Groups I and II were from study A, and IX was from study B. [b] Total residue was determined by combustion. [c] Percent total ^{14}C recovered = ppm of ^{14}C in final extract/ppm total residue. [d] Unchanged salinomycin was determined by HPLC. [e] Mean ± SD of four determinations per sample for study A and 3 determinations per sample for study B. [f] Control chickens.

Table IX. Percent Inhibition[a] of Rubidium Binding at pH 11.0 and 9.5 by Competing Cations in Liver Extracts from Treated Chickens and Control Liver Spiked with Salinomycin

	pH 11.0			pH 9.5		
displacing cation	salinomycin standards[b]	male liver	female liver	salinomycin standards[c]	male liver	female liver
Rb^+	23.9 ± 6.29	10.2 ± 7.75	14.5 ± 6.87	18.6 ± 2.75	4.6 ± 0.15	7.7 ± 2.00
K^+	43.2 ± 5.39	16.1 ± 3.72	21.9 ± 0.76	30.7 ± 1.88	8.1 ± 0.15	11.4 ± 1.87
Na^+	4.7 ± 2.61	3.0 ± 2.76	−0.9 ± 4.75	2.8 ± 0.83	2.4 ± 0.49	0.1 ± 0.56
Ca^{2+}	2.9 ± 3.49	6.4 ± 8.75	−2.9 ± 4.07	2.0 ± 1.70	3.0 ± 0.64	2.5 ± 2.35
Mg^{2+}	3.4 ± 3.42	4.1 ± 0.21	−2.7 ± 3.08	0.9 ± 1.90	3.5 ± 0.42	0.8 ± 1.22

[a] Percent inhibition = (^{86}Rb bound without cation − ^{86}Rb bound with cation)/^{86}Rb bound without added cation × 100. Values are means ± SD of triplicate determination and are corrected for the binding obtained with a 0 ppm salinomycin standard. [b] Salinomycin standards at 0.116, 0.342, and 1.902 ppm. [c] Salinomycin standards at 1.285 and 1.943 ppm.

ards was >90% of [^{14}C]salinomycin added and for the treated samples ranged from 56.0% to 80.7% of the total drug residue level. The difference in ^{14}C recoveries between treated samples and the standard samples would be expected, since salinomycin is extensively metabolized in the animal resulting in metabolites with a wide array of polarities. Since nonextractable residue in chicken liver accounts for 10% of the total salinomycin residue in chicken liver (data not presented), the extraction method accounted for a significant portion of the total extractable drug residue, which allowed us to assess the ability of the salinomycin metabolites to bind ^{86}Rb. With the ^{22}Na binding method of Fahim and Pressman (1981) for measuring monensin levels in tissues, a methanol extract of tissue is partitioned into heptane resulting in a more selective extraction of parent compound than of metabolites.

The ^{86}Rb binding data show a direct relationship between unchanged salinomycin levels or total drug residue levels and amount of ^{86}Rb bound. The ^{86}Rb binding value obtained for the 0 ppm salinomycin standard was especially low and would be comparable to values obtained without added tissue extract. The low blank values and low variability of the ^{86}Rb binding values obtained here are in direct contrast to the data obtained by Donoho (1984) and Pressman and Fahim (1983) for ^{22}Na binding in tissue extracts. Livers from treated groups that had the highest levels of total drug residue and unchanged salinomycin residue had the highest levels of Rb binding. However, unchanged salinomycin did not account for all of the ionophoric activity of the treated liver samples. From the ^{86}Rb binding data it is apparent that metabolites of salinomycin possess some ionophoric activity, since unchanged salinomycin is a minor fraction of the total salinomycin residue in liver and its tissue levels could not account for the observed levels of Rb binding. The ionophoric activities obtained from all of the treated samples are well below that obtained with unchanged salinomycin at 1 ppm. It can be extrapolated that approximately 20% of the extractable salinomycin residue possesses ionophoric activity or that tissue metabolites have lower binding affinities than that of unchanged salinomycin. The data of Miyazaki et al. (1976) show that salinomycin derivatives vary in their ability to bind radioactive cations and inhibit microbial growth.

The ^{86}Rb binding assay was developed in order to measure ionophoric activity of unchanged salinomycin. ^{86}Rb was the cation of choice for the method, and maximal binding of ^{86}Rb by salinomycin occurs at pH 11. It was important to determine whether the tissue extract from treated chickens exhibits an assay pH optimum and cation selectivity similar to that of unchanged drug. Therefore,

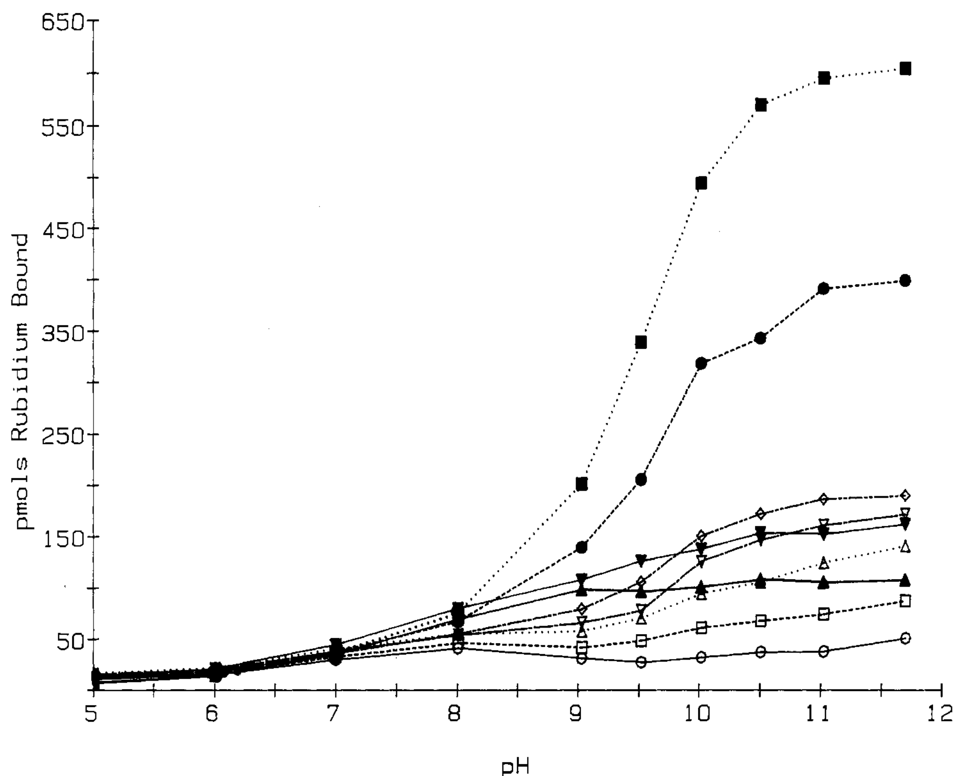

Figure 4. Effect of assay pH on rubidium binding in extracts of control liver spiked with 0 ppm (○), 0.10 ppm (□), 0.21 ppm (△), 0.32 ppm (▽), 0.45 ppm (◇), 1.09 ppm (●), and 1.99 ppm (■) [^{14}C]salinomycin and of liver from male (▲) and female (▼) chickens dosed with [^{14}C]salinomycin.

after the individual liver samples were assayed for ionophoric activity, the male and female liver homogenates from the group of chickens fed salinomycin alone were pooled, and then the effect of assay pH and cation selectivity was determined on ^{86}Rb binding by tissue extracts. The pooled, male and female, liver homogenates were run concurrently with control homogenate spiked with [^{14}C]-salinomycin. The data are presented in Figure 4. The levels of total drug residue were 1.128 ppm for the male and 1.770 ppm for the female liver samples. Recoveries of total ^{14}C radioactivity were 62% for the male sample, 66% for the female sample, and ranged from 89% to 93% for the [^{14}C]salinomycin standards. For the salinomycin standards, ^{86}Rb binding plateaued at pH 11 and no binding was observed at an acidic or neutral pH. Binding of ^{86}Rb increased linearly with increasing concentration of salinomycin, and all of the [^{14}C]salinomycin standards exhibited a similar pH profile. For the male and female samples, binding was maximal at pH 11 and generally was less at decreasing pH. At pH 11 the level of ^{86}Rb binding was slightly lower for the male than for the 0.21 ppm standard, and at pH 11 the level of ^{86}Rb binding was slightly lower for the female than for the 0.32 ppm standard. The pH profile for the treated samples was qualitatively similar to the pH profile of the [^{14}C]salinomycin standards.

The effect of adding 10 nmol of Rb^+, K^+, or Na^+ or 5 nmol of Ca^{2+} or Mg^{2+} on the binding of 10 nmol of ^{86}Rb at pH 9.5 and 11 was determined for the male and female liver homogenates and for control homogenates spiked with [^{14}C]salinomycin. The data in Table IX show that the order of cation selectivity for binding by salinomycin in the liver extracts at pH 9.5 and 11 was as follows: $K^+ > Rb^+ > Na^+, Ca^{2+}, Mg^{2+}$. The extent of inhibition of Rb^+ and K^+ on ^{86}Rb binding for the treated samples was not as great as for the salinomycin standards; however, the treated samples exhibited the same order of cation selectivity as that of unchanged salinomycin. The ^{86}Rb binding assay described here is relevant for the determination of ionophoric activity in livers of chickens fed salinomycin. Rubidium binding increases with increasing concentration of salinomycin, and the ionophoric activity of liver from treated chickens is qualitatively similar to that of unchanged salinomycin. Even though the total drug residue levels reported here are at the tolerance limit of 1.8 ppm for liver, the salinomycin residue would be expected to have no toxicologic consequences, since the extractable residues represent 90% of the total drug residue in liver and only approximately 20% of the extractable residue possesses ionophoric activity.

Registry No. Salinomycin, 53003-10-4; roxarsone, 121-19-7; lincomycin, 154-21-2; bacitracin MD, 70356-93-3.

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Purification and Analysis of Versiconal Hemiacetal Acetate and Versicolorin A Using Low-Pressure Liquid Chromatography and High-Performance Liquid Chromatography

Julia A. Billington and Dennis P. H. Hsieh*

To elucidate the enzymatic conversion of versiconal hemiacetal acetate (VHA) in the biosynthesis of aflatoxin B_1 (AFB) by *Aspergillus parasiticus*, methods were adapted for the purification and analysis of this and the related intermediate versicolorin A (VA). Following production of these biosynthetic intermediates, VHA and VA were purified on a low-pressure silica gel column developed with dichloromethane-based solvent systems. Both compounds were pure as determined by thin-layer chromatography in several solvent systems. VHA was quantitated with molar extinction coefficients established in an acidified ethanol solution. Using reversed-phase high-performance liquid chromatography (HPLC), two separation systems were developed. An isocratic HPLC solvent system consisting of methanol/acetonitrile/0.053 N glacial acetic acid in water (42.5:42.5:15) (q.s.) was successful in separating VHA, VA, and related compounds. The retention times for VHA and VA were 2.31 and 3.26 min, respectively. An isocratic solvent system of acetonitrile/0.053 N glacial acetic acid in water (55:45) was required to separate a product of the enzyme-catalyzed reaction, tentatively identified as versicolorin C (VC), from VA. With this solvent system, the retention times for VHA, VC, and VA were 3.72, 7.12, and 7.82 min, respectively. This HPLC system offers a rapid and sensitive assay for VHA conversion in an *A. parasiticus* cell-free extract.

Versicolorin A (VA), sterigmatocystin (ST), and aflatoxin B_1 (AFB) are biogenetically related mycotoxins formed by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Singh and Hsieh, 1977). They have been found carcinogenic in laboratory animals (Hendricks et al., 1980; Wogan et al., 1974), and AFB is implicated in the etiology of human liver cancer in some African and Asian countries (Van Rensburg et al., 1985). The health significance of AFB has prompted active investigation into its biosynthesis in the toxigenic fungi for possible control of the production of these mycotoxins in foodstuffs such as grains and nuts (Bennett and Christensen, 1983). With use of radiotracer techniques in conjunction with mutants and metabolic inhibitors, and also cell-free studies, it has been established that the sequence of biosynthesis for known intermediates is acetate → norsolorinic acid → averantin → averufanin → averufin → versiconal hemiacetal acetate → versicolorin A → sterigmatocystin → *O*-methylsterigmatocystin → aflatoxin B_1 (Anderson and Dutton, 1980; Bennett et al., 1980; Bhatnagar et al., 1987; Cleveland et al., 1987; Hsieh et al., 1973, 1976b; Hsieh and Mateles, 1970; Lee et al., 1976; Lin et al., 1973; McCormick et al., 1987; Singh and Hsieh, 1976, 1977; Wan and Hsieh, 1980) (Figure 1). Of particular interest in this pathway are the enzymatic reactions involved in the conversion of VHA to VA (Wan and Hsieh, 1980; Anderson and Dutton, 1980), thereby creating the bisfuran portion of the molecule (Hsieh et al., 1976a) and converting a compound with negligible mutagenicity to one with substantial mutagenicity (Wong et al., 1977). As part of the effort to monitor and characterize these enzyme reactions, methods for the efficient production, purification, and analysis of these compounds were developed.

Department of Environmental Toxicology, University of California, Davis, California 95616.

MATERIALS AND METHODS

Organisms. Both an aflatoxin-producing strain, *A. parasiticus* ATCC 15517, and a versicolorin A accumulating mutant, *A. parasiticus* ATCC 36537 (Lee et al., 1975), were used in this study. The latter was suppled by Dr. J. W. Bennett of Tulane University, New Orleans, LA. Fungal conidia were harvested from well-sporulated cultures on potato dextrose agar (Difco Laboratories, Detroit, MI) and stored at 4 °C in a 0.01% sodium lauryl sulfate solution for up to 3 months.

Chemicals and Solvents. The dichloromethane, hexane, and methanol used for low-pressure liquid chromatography were Baker Resi-Analyzed grade (J. T. Baker Chemical Co., Phillipsburgh, NJ). The formic acid was ACS reagent grade supplied by Allied Chemical (Morristown, NJ). Acetic acid was ACS reagent grade obtained from Fisher Scientic Co. (Fair Lawn, NJ). The water and methanol used for HPLC analysis were HPLC grade from Fisher, and HPLC-grade acetonitrile was obtained from J. T. Baker. Dichlorovos was obtained from Chem Service (West Chester, PA).

Production and Extraction of VHA and VA. VHA is accumulated in the mycelium of *A. parasiticus* when the aflatoxin-producing cultures are treated with the pesticide and cholinesterase inhibitor dichlorovos (phosphoric acid, 2,2-dichloroethenyl dimethyl ester) (Yao and Hsieh, 1974). To produce VHA, 1×10^7 *A. parasiticus* ATCC 15517 conidia were added to 100 mL of minimum mineral (MM) medium (Adye and Mateles, 1964) in 500-mL baffled flasks (Bellco Glass Inc., Vineland, NJ) and incubated at 30 °C, 100 rpm for 24 h and then at 200 rpm for 24 h. The mycelium was then transferred to 100 mL of

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