Salinomycin Residues and Their Ionophoricity in Pig Tissues

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The effect of pretreatment with medicated feed on [^{14}C]salinomycin residue levels in pig tissues was studied. Pigs were fed unmedicated feed or feed medicated with salinomycin at 41 ppm in the diet for 29 days and then dosed with [^{14}C]salinomycin for 8 days. Total drug residue levels were below quantifiable limits of detection of kidney, fat, and muscle but at the tolerance limit of 1800 ppb for liver. In liver, pretreatment tended to lower total residue levels, and unchanged [^{14}C]salinomycin accounted for <1% of the total drug residue. Approximately 15–20% of the total drug residue in liver was bound. Ionophoric activity in extracts of livers from the treated pigs was minimal, and only 2 of the 12 treated samples had ionophoric activity more than twice that obtained from the controls.

Sodium salinomycin (Figure 1) is a polyether antibiotic possessing anticoccidial and growth-promoting activity (McClure et al., 1980). Recently, sodium salinomycin has been shown to improve efficiency of gain in swine without affecting feed intake (Lindemann et al., 1985). No compounds from the ionophoric antibiotic class of compounds are currently approved for use in swine diets in the United States.

In an earlier residue study conducted at our facility, total drug residue levels of salinomycin in livers from pigs dosed with [^{14}C]salinomycin at 110 ppm in the diet were above the tolerance limit of 1800 ppb at zero withdraw (8 h after the last dose). By 4 days after dosing, total drug residue levels were well below the tolerance limit. The extractable residue depleted from the liver faster than the bound residue, and by 4 days after dosing the bound residue accounted for approximately 70% of the total residue. However, the bound residues were below the tolerance limit. These data would preclude a zero withdraw period for pigs dosed with salinomycin at 110 ppm in the diet.

Salinomycin is efficacious at a level of 27.5 ppm in the diet, and at this dosage level the residue levels in liver should drop below the tolerance limit. Therefore, a residue study was conducted to satisfy the requirements for approval of salinomycin as a feed additive in swine diets. The pigs were dosed with [^{14}C]salinomycin at 41 ppm in the diet, which is 1.5 times the intended field use level. The results of the residue study are reported here.

MATERIALS AND METHODS

Salinomycin Standards. The standard substances were sodium salinomycin (926 mg of active drug/g) and ^{14}C-labeled sodium salinomycin ([^{14}C]salinomycin). The radiopurity of [^{14}C]salinomycin was >98% by normal-phase thin-layer chromatography in the following solvent systems: acetone–ethyl acetate–methylene chloride–acetic acid (33:33:33:0.5); 25% trimethylene in methanol–benzene–*n*-heptane (2:9:9); methylene chloride–methanol–ammonium hydroxide (95:5:0.5); methylene chloride–methanol–acetic acid (95:5:0.5). The internal standard (IS) was sodium narasin (934 mg of active drug/g), obtained from Eli Lilly and Co., Indianapolis, IN.

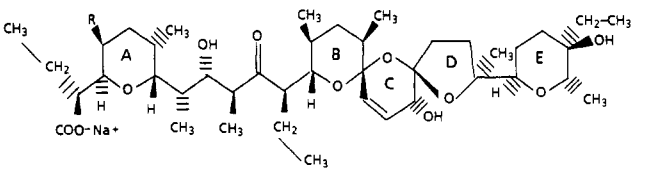

Figure 1. Structure of sodium salinomycin and sodium narasin.

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 PT20ST probe generator (Brinkmann Instruments, Inc., Westbury, NY); Tri-Carb Model 306 oxidizer, Model 4530 liquid scintillation spectrometer, Carbo-Sorb, Permafluor 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 and Vac-Elut vacuum manifold (Analytichem International, Harbor City, CA); a 24-position nitrogen evaporator (Organomation, South Berlin, MA).

Reagents and Solvents. Reagents for the ionophore assay were purchased from Sigma Chemical Co., St. Louis, MO. Rubidium-86 chloride (^{86}Rb, specific activity 250 μCi/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 Chemical Co., Phillipsburg, NJ, or Burdick and Jackson, Muskegon, MI.

Preparation of Dose Formulation. Each of two different batches of dose formulation were prepared approximately 2 weeks before dosing. The dose formulations were prepared by dissolving sodium salinomycin in 50 mL of a solution of sodium [^{14}C]salinomycin in ethanol in a 500-mL flask. Anhydrous lactose was added to a 2-L beaker, and the beaker was placed in a warm water bath. The [^{14}C]salinomycin solution was added to the lactose, the flask was rinsed with methanol, and the rinse was added to the lactose. The [^{14}C]salinomycin/lactose mixture in solvent was stirred constantly with a glass rod under a stream of nitrogen until the solvent was nearly evaporated. The mixture was then spread onto filter paper, covered loosely with aluminum foil, and allowed to air-dry overnight under a hood at room temperature. The mixture was then ground to a fine

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0021-8561/90/1438-1029$02.50/0

powder with a mortar and pestle and spread onto filter paper. Portions of the [^{14}C]salinomycin/lactose formulation were then removed for analysis. The first batch of dose formulation totaled 210 g, and the second batch totaled 121 g.

The final concentration, determined by high-performance liquid chromatography (HPLC), of [^{14}C]salinomycin in the dose formulations was 44.49 mg of [^{14}C]salinomycin/g of formulation for the first batch and 38.94 mg of [^{14}C]salinomycin/g of formulation for the second batch. The specific activity of the dose formulations was 3.066 μCi/mg of [^{14}C]salinomycin for the first batch and 2.773 μCi/mg of [^{14}C]salinomycin for the second batch. The radiochemical purity was >98% by thin-layer chromatography in the same systems used for the salinomycin standards. One day before dosing, the required amounts of [^{14}C]salinomycin/lactose formulation for each pig and dose were weighed and added to 1-oz. clear gelatin capsules for storage at room temperature.

Animal Treatment. Crossbred swine, obtained by mating crossbred gilts to Duroc, Landrace, Hampshire, or Yorkshire boars, were used for the study. The in-life portion of the study was conducted at Colorado State University, Ft. Collins, CO, and all sample analyses were done at the A. H. Robins Research Center, Richmond, VA. The study was conducted in two phases, staggered by 2 weeks. The female pigs were run in the first phase, and the male pigs were run in the second phase. A total of 12 male and 12 female pigs were used for the study. At the start of the study (day 1) the female pigs were 13 weeks and 3 days of age with an average body weight of 45.8 kg; the male pigs were approximately 14 weeks of age with an average body weight of 48.8 kg. The basal diet was unmedicated 16% Hog Grower Mash (Denver Feed Co., Commerce City, CO), formulated to meet or exceed nutrient specifications of swine (NRC, 1979).

On days 1–29 the pigs (five males and five females) in treatment group I were fed unmedicated basal ration, ad libitum; the pigs (five males and five females) in treatment group II were fed basal ration medicated with sodium salinomycin at 41 ppm, ad libitum. The medicated basal ration was prepared by mixing basal ration with medicated premix (30 g of sodium salinomycin/lb of premix). Treatment group III (controls) consisted of two males and two females. Pigs from group III were fed unmedicated basal ration throughout the study, and on day 29 one male and one female pig were culled from the study and the two remaining pigs were killed. On days 1–19 the pigs were housed in group pens with concrete floors. On days 20 until slaughter, the pigs in group I and II were individually housed in metabolism cages, designed for the separate collection of urine and feces, containing an automatic watering device and stainless steel feed bowl. On days 20–29 the control pigs were housed in a group pen in a room separate from the treated pigs.

The pigs were adapted to a meal feeding regimen on days 27–29. On day 29 two males and two females were culled from each of groups I and II on the basis of body weight and/or failure to adapt to the meal feeding regimen, leaving three males and three females in each of groups I and II. On days 30–37 the pigs in groups I and II were dose with [^{14}C]salinomycin at 41 ppm in the diet by a meal feeding regimen at 12-h intervals. At each meal, the amount of feed required was divided into two equal portions. The first half of the meal was mixed 1:1 with tap water in the feed bowl. The [^{14}C]salinomycin/lactose capsule was opened, and the entire contents and emptied capsule were mixed into the feed and immediately offered to the pig. After the pig had completely consumed the first half of the meal, the remaining half (unmedicated feed) of the meal was offered. During the [^{14}C]salinomycin dosing period, the daily amount of feed offered to each pig was calculated by multiplying body weight on day 29 by average daily feed consumption expressed as a percentage of body weight during days 21–27. On day 29 the average body weight of the male pigs was 75.7 kg and of the female pigs was 69.1 kg.

Each pig was stunned with a captive bolt pistol and then killed by exsanguination at 8.0 h (zero time withdraw) after its final dose of [^{14}C]salinomycin. The times of administration of the last three doses of [^{14}C]salinomycin were staggered, to ensure that all pigs were killed at 8 h after the last dose.

Tissue Collection and Homogenization. At the time of slaughter, the following tissues were collected: readily available subcutaneous back fat and kidney fat, longissimus muscle, the entire liver, and both kidneys. The tissues were rinsed with water, blotted dry, weighed, placed in a labeled plastic bag, immediately placed on dry ice, and stored frozen until analysis.

The frozen tissues were partially thawed by placing them in a cold room overnight and then completely thawed in cold water. The entire liver, both kidneys, and fat samples were separately homogenized in a blender, and the entire muscle sample was ground three times through a meat grinder. Approximately 50 g of neat homogenate of liver, kidney, muscle, and fat was accurately weighed and homogenized with water (tissue/water = 1/2.33, w/v) in a Polytron tissue homogenizer to produce a 30% homogenate. The homogenates in water were stored frozen until analysis.

Total Drug Residue Analysis. The tissue homogenates were analyzed for total drug residue by combusting 0.5-mL portions (equivalent to 150 mg of wet tissue) of homogenate in water. Tissue samples from control pigs were used to establish the level of background radioactivity. The quantifiable limits of detection for total drug residue were twice background or 85 ppb for muscle, 91 ppb for fat, 85 ppb for kidney, and 84 ppb for liver from the females and were 111 ppb for muscle, 149 ppb for fat, 107 ppb for kidney, and 104 ppb for liver from the males.

Total drug residue levels in all of the muscle, fat, and kidney samples were below the quantifiable limits of detection. The tolerance limits for total drug residue in muscle, fat, and kidney are 600, 2400, and 2400 ppb, respectively.

Unchanged Salinomycin Analysis. The concentration of unchanged [^{14}C]salinomycin was determined in the liver homogenates by an HPLC method described by Dimenna et al. (1989), which was used for the analysis of salinomycin in chicken liver. For the standard curve, control liver homogenate was spiked with salinomycin at 0, 5, 10, 20, 40, 100, and 200 ppb. All samples were spiked with IS at 150 ppb. Quality control (QC) homogenates were spiked with salinomycin at 30, 60, and 90 ppb at approximately the same time the tissue samples were removed from the pigs. The QC samples were stored frozen and assayed concurrently with the study samples. Muscle, fat, and kidney samples were not assayed for unchanged salinomycin, because the total residue levels were very low and unchanged drug would likely have been below the quantifiable limit of detection for the method.

Bound Residue Analysis. Duplicate 3.3-mL portions (equivalent to 1 g of tissue) of liver homogenate were added to tared tubes and extracted with 20 mL of methanol by shaking for 30 min. The samples were centrifuged, and the methanol extract was decanted into a 50-mL volumetric flask. The precipitate was extracted an additional two times with 12 mL of methanol each time, and the three methanol extracts were combined and brought to volume with methanol. Ten-milliliter portions of the methanol extract were added to liquid scintillation vials and evaporated to dryness under a stream of nitrogen. The precipitate remaining after methanol extraction was extracted two times with 15 mL of acetone each time followed by two additional extractions with 15 mL of ethyl acetate each time. The two acetone extracts were combined as were the two ethyl acetate extracts and evaporated to dryness under a stream of nitrogen. Extractable radioactivity was determined in the extracts by counting in 10 mL of Insta-Gel two times for 10 min each.

For the determination of the bound residue the precipitate remaining after solvent extraction was assayed for total radioactivity. The precipitate was dried in vacuo at 40 °C. The dried tubes were weighed. The precipitate was divided approximately in half, and each half was added to a tared combustion cone for oxidation. The precipitate (plus combustion cone) was weighed and then directly combusted. The $^{14}CO_2$ was trapped in Carbo-Sorb and counted twice for 10 min each in Permafluor V.

Ionophoric Activity Determination. Ionophoric activity was determined in extracts of the liver homogenates by a radiolabeled rubidium (^{86}Rb) binding method described by Dimenna et al. (1989). For the standard curve, control liver homogenate was spiked with [^{14}C]salinomycin at 0, 63, 263, 387, 667, 1333, 1951, and 2929 ppb.

Table I. Disposition of Salinomycin Residue in Liver

group[a]	pig no.	sex	residue level, ppb			
			total	salinomycin	extractable	bound
I	1	F	2011	5	1675	393
	2	F	1367	6	1281	214
	3	F	1458	6	1214	280
	26	M	2754	18	2405	392
	27	M	2622	7	2265	400
	29	M	2823	5	2508	392
mean			2173	8	1891	345
II	6	F	1852	5	1556	272
	9	F	1061	6	893	189
	10	F	971	7	883	193
	31	M	2548	12	2187	428
	32	M	1255	<5	963	377
	35	M	1309	<5	941	337
mean			1499	8	1237	299

[a] Pigs from group I were fed unmedicated feed before being dosed with [^{14}C]salinomycin at 41 ppm in the diet for 8 days. Pigs from group II were fed feed medicated with salinomycin at 41 ppm in the diet for 29 days before being dosed with [^{14}C]salinomycin at 41 ppm in the diet for 8 days.

RESULTS AND DISCUSSION

The residue levels obtained in liver are presented in Table I. Of all tissues assayed for total drug residue, residue levels were higher in liver. Total drug residue levels were higher in the male livers than in the female livers. However, due to the small number of animals used in the study, the significance of this effect was not truly evaluated. Pretreatment of the pigs with salinomycin in the diet tended to decrease mean total drug residue levels in liver, although considerable overlap in residue levels existed between the two groups. Dosing pigs for an 8-day period was sufficient to achieve steady-state levels of total drug residue in liver. Total drug residues do not accumulate in the liver, since pretreatment of the animals with unlabeled drug had no significant effect on residue levels.

The residue levels obtained in liver in this study show a dose–response relationship when compared with the data obtained in the earlier residue depletion study. Since the dose level was 1.5 times the intended field use level of 27.5 ppm, residue levels in liver would likely be lower in pigs dosed at the use level.

The liver samples were assayed for unchanged [^{14}C]salinomycin by HPLC, and it accounted for <1% of the total drug residue in all samples. Unchanged salinomycin would not be a suitable marker compound for total drug residue in liver. In swine salinomycin is extensively metabolized, resulting in numerous metabolites in the liver, none of which have been identified or are present in sufficient quantity to be useful as a marker compound (data not presented).

Pretreatment with salinomycin in the feed had no effect on the level of bound residue in the liver. From the earlier residue depletion study, the elimination half-life of the bound residue was estimated to be 5.5 days. The amount of [^{14}C]salinomycin-related bound residue in the liver should approach saturation by pretreating pigs with unlabeled salinomycin for 29 days, resulting in lower bound residue levels than in nonpretreated pigs. However, the levels of bound residue obtained in this study were not affected by treatment, suggesting that the bound residue was at steady state under the two dosing regimens. The levels of bound residue obtained here represented approximately 15–20% of the total drug residue.

Since the levels of total drug residue in livers from half of the pigs were above the tolerance limit of 1800 ppb, the biological significance of these residue levels was assessed by determining the ionophoric activity of the liver extracts. Ionophores, such as salinomycin, exert their pharmacologic and toxicologic activity by forming electrically neutral zwitterionic complexes with cations and transporting these ions across biological membranes, thereby disturbing numerous intracellular functions (Reed, 1982). An ^{86}Rb binding assay, described by Dimenna et al. (1989), was developed for the determination of ionophoric activity in extracts of liver tissue from animals dosed with salinomycin. 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.

Table II. Drug Residue Levels in Liver vs Amount of ^{86}Rb Bound by Liver Extracts

spiked std,[c] ppb	total drug residue, ppb	recovered, ppb	pmol ^{86}Rb bound
0	0	0	23.3
63	63	47	41.0
263	263	197	116.0
387	387	312	169.4
667	667	524	283.1
1333	1333	1031	541.5
1951	1951	1507	760.8
2929	2929	2409	1177.7

pig no.	group[a]	sex	total drug residue, ppb	recovered,[b] ppb	pmol ^{86}Rb bound
1	I	F	2011	1228	51.0
2		F	1367	1015	56.4
3		F	1458	867	51.9
26		M	2754	1726	114.9
27		M	2622	1650	66.4
29		M	2823	1829	56.3
6	II	F	1852	1207	52.1
9		F	1061	675	61.4
10		F	971	670	60.5
31		M	2548	1714	87.0
32		M	1255	720	43.2
35		M	1309	716	53.9
45	III	F	0	0	32.9
60		M	0	0	36.4

[a] Pigs from group I were fed unmedicated feed before being dosed with [^{14}C]salinomycin at 41 ppm in the diet for 8 days. Pigs from group II were fed feed medicated with salinomycin at 41 ppm in the diet for 29 days before being dosed with [^{14}C]salinomycin at 41 ppm in the diet for 8 days. Pigs from group III were controls and fed unmedicated feed throughout the study. [b] Amount of residue recovered from the extraction method used for the ionophore analysis. [c] Control liver homogenates spiked with [^{14}C]salinomycin at the stated concentrations.

The ^{86}Rb binding values obtained from the treated liver samples are presented in Table II. Control liver homogenates from the study 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 extract and raned from 74 to 82% of [^{14}C]salinomycin added for the standard samples and 71 to 79% of the extractable drug residue from the treated samples. The levels of ^{86}Rb binding were directly related to the levels of unchanged [^{14}C]salinomycin in the standard samples. However, of the 12 liver samples from treated pigs, only two samples (pigs 26 and 31) had ^{86}Rb binding values more than twice that obtained from livers of the two control pigs. The total drug residue level in liver of pig 26 was 2754 ppb, and its ^{86}Rb binding value was similar to that obtained with the 263 ppb [^{14}C]salinomycin standard. Therefore, it is apparent that the drug residue in liver

from this sample had less than 10% of the ionophoric activity of an equivalent amount of salinomycin. This degree of ionophoric activity represents the maximum observed, while that obtained with the remaining samples was nearly negligible. The low ^{86}Rb binding levels in the treated samples are likely due to the very low levels of unchanged salinomycin in these samples and the high degree of polarity of the salinomycin metabolites in pig liver (data not presented) resulting in a loss of ionophoric activity. The levels of ^{86}Rb binding obtained with the two control samples were between those obtained with the 0 and 63 ppb [^{14}C]salinomycin standards.

CONCLUSIONS

Even though the residue levels in liver are at the tolerance limit of 1800 ppb, their toxicological consequences would be minimal as reflected by their lack of ionophoric activity, since only 2 of the 12 liver samples from treated pigs had ionophoric activity greater than twice that observed with the control samples. Of these two liver samples, the extractable residue had less than 10% of the ionophoric activity of an equivalent amount of salinomycin.

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Received for review September 15, 1989. Accepted January 17, 1990.

Registry No. Salinomycin, 53003-10-4.

Structure Elucidation of the Product Prepared from the Reaction of 3-Methyl-5,6-dihydro-2(1*H*)-pyrazinone and Ketones or Aldehydes

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The condensation products prepared from reactions of 3-methyl-5,6-dihydro-2(1*H*)-pyrazinone (1) and ketones or aldehydes had previously been assigned the structures 5-alkyl-3-methyl-2(1*H*)-pyrazinones **3**. Current work revealed that the formulas should be revised to 6-alkyl-3-methyl-2(1*H*)-pyrazinones **2**.

Some alkoxy- and (alkylthio)pyrazines derived from 2(1*H*)-pyrazinones (Masuda et al., 1981; Masuda and Mihara, 1986) showed high potential use as flavor ingredients (Fors, 1983). Although 5-alkyl-3-methyl-2(1*H*)-pyrazinones **3** prepared from the reaction of 3-methyl-5,6-dihydro-2(1*H*)-pyrazinone (1) and ketones or aldehydes have been reported (Masuda et al., 1981), their chemical structures were not fully examined. Determination of the structure of these compounds by ^1H NMR spectra is not necessarily straightforward (MacDonald et al., 1976). We now report that NOE difference experiments and X-ray crystallography demonstrate that these compounds are actually 6-alkyl-3-methyl-2(1*H*)-pyrazinones **2** (see Scheme I).

EXPERIMENTAL SECTION

Instrumentation. The IR, ^1H NMR, and GC/MS were recorded on a Hitachi 260-10, a Bruker AM-400, and a Hitachi M-80B spectrometer, respectively. GC analyses were carried out on a Hewlett-Packard Model 5710A gas chromatograph equipped with a flame ionization detector and a fused-silica capillary column coated with Carbowax 20M or OV-101.

Synthesis of 3-Methyl-2(1*H*)-pyrazinone (2'). This compound was prepared by the method described by Masuda and Mihara (1986).

Derivation of 2-Isopropyl-5-methylpyrazine (5a) from 6-Isopropyl-3-methyl-2(1*H*)-pyrazinone (2a) (See Scheme II). Compound **2a**, prepared as described by Masuda et al. (1981) (2 g, 0.013 mol), and phosphorus oxychloride (20 g, 0.13 mol) were refluxed for 2 h. The reaction mixture was then gradually added to ice-cooled water (100 mL). The reaction mixture was adjusted to pH 8 with 50% NaOH. After the insoluble material was filtered off, the filtrate was concentrated under vacuum. The oily residue (2.3 g) was further distilled in vacuo to give 3-chloro-5-isopropyl-2-methylpyrazine (**4a**; 1.7 g, 0.01 mol); bp 68 °C (2 mm) (Karmas and Spoerri, 1952).

A dioxane solution (7 mL) of **4a** (1.7 g, 10 mmol) was hydrogenated in the presence of Pd/C (10%, 0.2 g) and sodium meth-

0021-8561/90/1438-1032$02.50/0