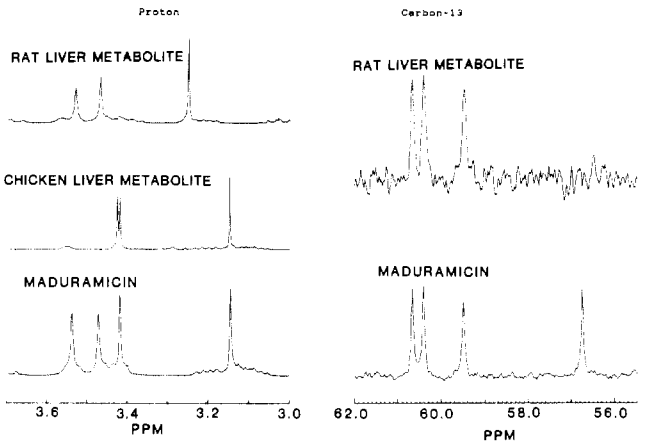

Figure 2. Comparison of the methoxy resonances of maduramicin and the rat liver metabolite in proton and carbon-13 NMR and of the chicken liver metabolite in proton NMR.

Table II. Selected Carbon-13 NMR Chemical Shifts[a] of Maduramicin[b] vs. Its Rat Liver Metabolite

carbon atom[c]	maduramicin	rat liver metabolite	change[d]
5	86.0	86.5	−0.5
6	82.5	82.5	0.0
39	37.4	40.2	−2.8
40	81.3	71.6	+9.7
41	86.6	87.7	−1.1
42	71.7	71.6	+0.1
44	60.6	60.7	−0.1
45	56.8	*e*	*e*
46	59.5	59.5	0.0
47	60.5	60.5	0.0

[a] In ppm relative to Me_4Si in deuteriobenzene. [b] Taken from Rajan et al. (1984). [c] See Figure 1 for carbon atom assignments. [d] (−) = downfield shift; (+) = upfield shift. [e] C-45 was absent from the metabolite spectrum.

ably these two singlets arise from the two methoxy groups on the unaffected portion of RLM, i.e. the A-ring (Figure 1).

Carbon-13 NMR provides conclusive evidence that the O-demethylation reaction producing the rat liver metabolite occurs at C-45 on the sugar moiety (Figures 1 and 2; Table II). In addition to the disappearance of the C-45 methyl carbon peak in the spectrum of the metabolite, the carbon atom on the other side of the relevant oxygen (C-40) is shifted upfield 9.7 ppm from 81.3 to 71.6 ppm (Table II). Also the two nearest neighbors to C-40 (C-39 and C-41) in the sugar moiety are shifted from 37.4 and 86.6 to 40.2 and 87.7 ppm, respectively (Table II). These observations are completely consistent with the changes in chemical shifts seen in the analogous carbon atoms in the comparison of maduramicin with CLM (Tsou et al., 1984) or in the comparison of the carbon-13 NMR spectra of cyclohexanol vs. cyclohexane methyl ether (Wehrli and Withlin, 1976). All other carbon nuclei in the metabolite give chemical shifts that are essentially indistinguishable from their equivalent nuclei in maduramicin.

Thus, rats treated orally with maduramicin either in feed at 5.2 ppm or by gavage with a single 1.0-mg dose efficiently produce a single major metabolite in the liver, which has been shown by carbon-13 and proton NMR and mass spectroscopy to represent an O-demethylation reaction at one of two methoxy groups of the sugar moiety. This is the first report of the involvement of a sugar moeity in the metabolism of a polyether carboxylate ionophore antibiotic. In contrast, metabolism of maduramicin in chickens involves O-demethylation of the A-ring. Metabolism examined to date of polyether anticoccidial compounds in mammals and chickens appears to primarily involve O-demethylation. In the case of maduramicin only the site of O-demethylation is different in rats vs. chickens.

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Registry No. Maduramicin, 79356-08-4; rat liver metabolite, 100702-75-8.

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Thin-Layer Bioautographic Assay for Salinomycin in Chicken Liver

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A thin-layer bioautographic method was developed for the determination of salinomycin in chicken liver. An aliquot of liver homogenate equivalent to 2 g of liver was extracted with isooctane. The extract was then purified on a silica gel column and chromatographed on silica gel thin-layer plates. The salinomycin zones were quantitated by a bioautography technique that makes use of *Bacillus subtilis*. The limit of detection was 25 ppb, and the assay response was linear from 25.0 to at least 200 ppb. The mean recovery of salinomycin from spiked samples was 108.2 ± 15.8% ($\bar{x}$ + SD).

INTRODUCTION

Sodium salinomycin is a polyether antibiotic that functions as an anticoccidial (Miyazaki et al., 1974) and that promotes growth (McClure et al., 1980). Its chemistry and biological activity have been previously described by Miyazaki et al. (1974).

Thin-layer bioautographic techniques that make use of *Bacillus subtilis* for the detection of monensin in animal tissues have been described by Donoho et al. (1967) and

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for the detection of lasolocid in animal tissues by MacDonald (1978). Heil et al. (1984) developed a thin-layer bioautographic technique that made use of *Bacillus stearothermophilus* for the semiquantitative detection of salinomycin in rabbit tissues. However, *B. stearothermophilus* can vary considerably in its sensitivity to salinomycin and is not as stable as *B. subtilis*. This paper describes a thin-layer bioautographic method that makes use of *B. subtilis* for the detection of salinomycin in chicken liver.

MATERIALS AND METHODS

Standards, Solvents, and TLC Plates. Sodium salinomycin (potency 970 μg/mg) was used as the reference standard. The solvents were of nanograde or HPLC grade, and water was deionized and distilled in glass (ddH_2O). The columns used for sample cleanup were packed with Sepralyte unbonded silica gel (40 μm, irregular particles; Analytichem International, Harbor City, CA). The columns were placed on a vacuum manifold (Vac-Elut, Analytichem International). The thin-layer chromatography (TLC) plates were glass Uniplate (20 × 20 cm) that were precoated with silica gel GF 250-μm thickness with a preabsorbent layer (Analtech, Inc., Newark, DE.) A tissue homogenizer (Polytron Model PT-35) with a probe generator (Model PT-20ST) purchased from Brinkman Instruments, Inc., Westbury, NY, was used for tissue sample homogenization.

Tissue. Livers from chickens fed unmedicated feed were obtained from the A. H. Robins Research Farm and from a local grocery store. These control liver samples were used to determine the standard curves.

Preparation of Bacterial Spore Suspension. *B. subtilis 6633* was grown on slants made from antibiotic medium no. 1 (Bacto Penassay Seed Agar, Difco). The slants were incubated for 18–24 h at 35 °C and refrigerated until needed. The bacteria were washed from the stock slants with 3 mL of sterile ddH_2O. The suspensions were then transferred to Roux bottles (one Roux bottle for each stock slant) containing 100 mL of Bacto Penassay Seed Agar and incubated for 7 days at 35 °C. The bacteria were washed from the agar surface with 17 mL of sterile ddH_2O into a 50-mL centrifuge tube, thoroughly suspended, and heated for 30 min at 65 °C. The bacterial suspensions were then centrifuged for 20 min at 1500 rpm, the supernatant was discarded, and the spores were resuspended in ddH_2O. Enough sterile ddH_2O was added to bring the level in the centrifuge tubes to the full mark. The contents of the tubes were mixed well and centrifuged for 20 min at 1500 rpm, and the supernatant discarded. The washings were repeated until the supernatant was clear. The pellets from all the centrifuge tubes were pooled into a sterile bottle that contained 30 mL of sterile ddH_2O per Roux bottle harvested. The spore suspension was heated for 30 min at 65 °C and stored cold for up to 6 months.

Bioautography Media. Bioautography medium no. 1 consisted of the following dissolved in 7 L of ddH_2O: 17.5 g of yeast extract (BBL), 70.0 g of glucose (Baker Chemical Co.), 105.0 g of Agar-Bacto (Difco), 3.15 g of monobasic potassium phosphate, 4.83 g of dibasic potassium phosphate. Bioautography medium no. 2 consisted of the following dissolved in 7.5 L of ddH_2O: 18.75 g of yeast extract, 75.0 of glucose, 112.5 g of Agar-Bacto, 3.39 g of monobasic potassium phosphate, 5.19 g of dibasic potassium phosphate, 75.0 g of magnesium chloride hexahydrate. The pH of both media was adjusted to 5.4 with 3 N HCl. The tetrazolium chloride overlay solution (dissolved in 100 mL of sterile ddH_2O) consisted of 0.2 g of 2,3,5-triphenyltetrazolium chloride and 0.4 g of dextrose.

Table I. Regression Analysis of the Standard Curves Run in Duplicate over 5 Consecutive Days

day	intercept	slope	correln coeff
1	−10.5855	11.4872	0.9957
	−7.5326	14.9500	0.9993
2	−6.9876	15.6100	0.9963
	−7.3958	9.4409	0.9947
3	−7.1089	9.8130	0.9984
	−6.6164	9.5007	0.9983
4	−8.2917	10.2349	0.9953
	−9.9890	11.2580	0.9904
5	−9.5999	11.4640	0.9857
	−5.4908	9.2084	0.9985
mean	−7.9598	11.2967	0.9953
SD	1.6290	2.2700	0.0042
% CV	20.5	20.1	0.4

Table II. Determination of Salinomycin in Chicken Liver Samples Spiked with Various Amounts of Salinomycin

	salinomycin found			
salinomycin added, ppb	no. 1, ppm	no. 2, ppm	mean, ppm	as % of salinomycin added
32	30	29	30	94
32	51	43	47	147
32	36	35	36	113
32	36	38	37	116
32	32	34	33	103
48	52	46	49	102
48	55	57	56	117
48	56	58	57	119
48	56	50	53	110
48	50	50	50	104
140	145	137	141	101
140	194	204	199	142
140	133	143	138	99
140	168	171	170	121
140	114	124	119	85
180	181	178	180	100
180	165	187	176	98
180	194	198	196	109
180	162	180	171	95
180	165	157	161	89
mean				108.2
SD				15.8
% CV				14.6

Preparation of Spiking Standards. Solutions of sodium salinomycin to be used for tissue spiking were prepared in methanol at each of the following concentrations: 0, 0.5, 1.0, 2.0, 4.0 μg/mL. Liver homogenates spiked with these solutions produced a standard curve with tissue concentrations of salinomycin of 0, 25, 50, 100, and 200 ppb.

Extraction. The entire liver was cut into small pieces, weighed, and homogenized in water (tissue/water, 20/80, w/v). Duplicate, 10-mL aliquots equivalent to 2 g of tissue were spiked with salinomycin and stored frozen until analysis. The concentrations of salinomycin in the samples were unknown to the analyst at the time the samples were assayed. Analysis of these "blind" samples was completed within 2 weeks of their being frozen. The spiked homogenates were thawed in lukewarm water and transferred to 50-mL centrifuge tubes that had Teflon-lined caps. To develop a standard curve, 10 mL of control tissue homogenate was spiked with 100 μL of the spiking standards. The homogenate was extracted by being shaken with 40 mL of isooctane on a reciprocal shaker for 15 min. The samples were then centrifuged for 10 min at 2000 rpm, and the isooctane layer was transferred to 0.5-g silica gel columns. The columns were washed with 20 mL of methylene chloride. Salinomycin was eluted from the column with 6 mL of a solution of methylene chloride and methanol

Table III. Stability of Salinomycin in Spiked Chicken Liver Homogenates after Storage[a]

salinomycin added, ppb	salinomycin found		salinomycin added, ppb	salinomycin found	
	ppb	as % of salinomycin added		ppb	as % of salinomycin added
50	53	106	100	88	88
50	58	116	100	86	86
50	49	98	100	82	82
50	50	100	100	96	96

[a] Spiked liver homogenates were stored frozen for 3.5 months before being analyzed.

(9/1) and evaporated under nitrogen. The residue was transferred with methylene chloride to 1.5-mL autosampler vials, evaporated under nitrogen, and reconstituted in 25 μL of heptane.

Thin-Layer Chromatography. Portions of 20 μL of each solution were applied to the preabsorbent area of each TLC plate. A standard curve was determined for each plate. This spotting scheme allowed for two samples to be assayed in duplicate per plate. A 100-mL portion of the developing solvent (a solution of chloroform, methanol, and concentrated ammonium hydroxide, 95/5/0.5) was poured over saturation pads that lined a TLC developing tank. The plates were placed in the developing tank on Teflon blocks, care being taken not to allow the plates to touch the developing solvent. The plates were allowed to equilibrate for 30 min. They were then placed in the solvent and developed to approximately 2 cm from the top. The plates were air-dried, and the preadsorbent area was scraped off.

Bioautography. Agar media 1 and 2 were warmed in a steamer for 1.5 h. Agar medium no. 1 (100 mL) was poured into a bioassay tray (NUNC) and allowed to solidify with the lid off. The TLC plate was placed on top of the solidified agar. After agar medium no. 2 was cooled to 60 °C, *B. subtilis* spore suspension was added (1 mL of spore suspension/100 mL of agar medium). The spore–agar suspension (100 mL) was poured over the TLC plate, allowed to solidify with the lid on for approximately 30 min, and incubated for 17 h at 37 °C. Tetrazolium chloride overlay solution (25 mL) was poured over the plate, and the plates were incubated for 1 h at 37 °C.

Quantitation. The vertical and horizontal diameters of the zones were measured three times with a dial caliper. An equation for the least-squares regression line was calculated from the average zone diameters against known log concentrations of salinomycin. This equation was used to determine the concentration of salinomycin in unknown samples. The plates were photographed for permanent record storage.

RESULTS

Salinomycin gave sharp zones that were circular to ellipitical in shape with an R_f of approximately 0.7. The limit of detection was about 25 ppb salinomycin in the tissue. Roxarsone, virginiamycin, and zinc bacitracin (approved drugs commonly used in chicken feed) did not interfere with the determination of salinomycin and did not produce any bacterial inhibition zones. Monensin, but not lasolocid, had an R_f similar to that of salinomycin, which would interfere with its determination. TLC developing solvents that separated monensin from salinomycin interfered with the growth of the *B. subtilis* organism. For our purposes, however, this method would be used for assaying salinomycin in livers of chickens dosed with salinomycin only. Therefore, it was not necessary to have a method specific for salinomycin.

Standard curves were run in duplicate on 5 consecutive days to determine day-to-day reproducibility (Table I). The assay response was linear from 25 to at least 200 ppb. The amount of variation from day-to-day in the intercept and slope was acceptable for an assay of this type. The correlation coefficients were consistent and greater than 0.99 for all but one curve.

To determine the accuracy of the method, tissue homogenates were spiked in duplicate with salinomycin at concentrations that ranged from 32 to 180 ppb. The recovery ranged from 85% to 121% in all but two samples, the recoveries for which were 142% and 147% (Table II). These two samples were run on the same plate. The precision of the method was acceptable because all duplicate determinations were within ±10% of the mean of the duplicates.

To determine the stability of salinomycin after storage, tissue homogenates were spiked with salinomycin at 50 and 100 ppb and stored frozen for 3.5 months before being analyzed. The results of the analysis are presented in Table III. The storage conditions had no effect on the concentration of salinomycin in the liver homogenate.

DISCUSSION

The *B. subtilis* organism is more stable and consistent in its sensitivity to salinomycin over time than the *B. stearothermophilus* organism. Therefore, it was not necessary to pretest the *B. subtilis* suspension because its activity was consistent over a long time period.

Sample extraction with isooctane yields a cleaner extract and an easier workup than extraction with methanol or acetone, which are commonly used for extracting ionophores from tissues. Consequently, a larger proportion of the tissue extract can be spotted on the TLC plate. Nonpolar solvents such as hexane and isooctane are as effective as methyl *tert*-butyl ether and methanol in extracting unchanged salinomycin from the liver of a steer dosed with radioactive salinomycin, and all of the salinomycin residue in chicken liver is extractable (unpublished data). Therefore, isooctane is a suitable solvent for the extraction of unchanged salinomycin from chicken liver.

This thin-layer bioautographic method is certainly useful for the determination of the presence or absence of salinomycin in chicken liver. Quantitation could be improved by assaying control tissue spiked with a known concentration of salinomycin in conjunction with the unknown sample. If the recovery of salinomycin in the spiked control sample is unacceptable, the sample can be reassayed.

Registry No. Salinomycin, 53003-10-4.

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