Tissue Residues and Metabolism of Narasin in Chicken

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Chickens were fed 50 ppm [^{14}C]narasin rations. After 5 days, the chickens were sacrificed, and tissues were taken for assay. Tissues were assayed for total radioactivity by solubilization and liquid scintillation counting. The mean residue concentrations of narasin equivalents were 0.32 ppm in liver, 0.12 ppm in fat, 0.08 ppm in skin/fat, 0.04 ppm in kidney, and <0.04 ppm in muscle. Liver radioactivity was isolated by liquid−liquid extractions and preparative silica liquid chromatography (LC). Radioactivity in fat was isolated and assayed by high-performance liquid chromatography/electrospray-mass spectrometry/liquid scintillation counting (HPLC/ESMS/LSC). The radioactivity in fat was characterized as being predominately parent narasin. Excreta samples were also collected for isolation, quantification, and characterization of narasin and its metabolites. Fifteen metabolites and parent narasin were characterized from the excreta of chickens using HPLC/ESMS/LSC. These metabolites were predominately di- and trihydroxylated narasin and di- and trihydroxylated narasin B. These hydroxylated metabolites represented almost 50% of the total radioactivity in excreta. The chromatographic distribution and relative magnitude of radioactivity from liver and excreta were similar, suggesting that excreta metabolites are the same as those found in liver.

Keywords: *Narasin; residues; metabolites; mass spectrometry*

INTRODUCTION

Coccidiosis is a disease of poultry caused primarily by protozoa of the genus *Eimeria*. The most frequently used method for prevention of coccidiosis in broiler chickens is the inclusion of an ionophore antibiotic in their rations. Narasin, [(4*S*)-4-methylsalinomycin], a polyether ionophore, produced in fermentation by *Streptomyces aureofaciens* is used alone or in combination with nicarbazin for control of coccidiosis in broiler chickens (Berg and Hamill, 1978).

Although the metabolism of other ionophores has been published [semduracmicin in chicken (Lynch *et al*., 1992); monensin in steer, rat, and chicken (Donoho, 1984; Donoho *et al.*, 1978); and maduramicin in rat (Brown and Rajan, 1986), and turkey (Stout *et al*., 1991)] little has been published on the metabolism of narasin. Catherman *et al*. (1991) used [^{14}C]narasin to measure the clearance of radioactivity from blood and tissues of chickens and quail. However, no attempt was made to identify any narasin metabolites. In this study, [^{14}C]-narasin residues were determined in tissues, and excreta metabolites were isolated, characterized, and quantified using high-performance liquid chromatography/electrospray ionization-mass spectrometry (HPLC/ESMS) and liquid scintillation counting (LSC).

MATERIALS AND METHODS

Drugs and Chemicals. Narasin and [^{14}C]narasin were provided by Lilly Research Laboratories, Eli Lilly and Co. (Indianapolis, IN). [^{14}C]Narasin was made in fermentation by using *Streptomyces aureofaciens*. The labeled precursors were [1-^{14}C]propionate and [1-^{14}C]butyrate used in equimolar amounts. This placed the ^{14}C label in 10 positions on the molecule (Dorman *et al*., 1976) (Figure 1). All reagents were of analytical grade.

Dose Preparation and Administration. [^{14}C]Narasin with a specific activity of 1.14 μCi/mg and a radiochemical purity of 97% was combined with unlabeled narasin in an ethanolic solution to give [^{14}C]narasin with a specific activity of 0.44 μCi/mg. The ethanolic solution was applied to broiler grower rations at a concentration of 50 ppm.

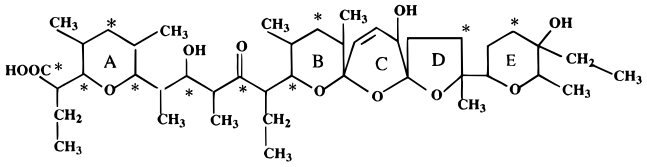

Figure 1. Structure of narasin. Asterisks denote the positions of ^{14}C labeling.

Animal Protocol. One day old Arbor Acre/Peterson cross chickens were identified by wing band at the time of arrival. They were housed under continuous light and fed from one day to three weeks of age on a starter broiler ration. From three weeks onward, they were fed a broiler grower ration. At three weeks of age, five chickens (three male and two female) were selected and weighed. The three males were assigned to one pen and the two females were housed in another pen. At six weeks of age, the chickens in these two pens were allowed *ad libitum* access to feed containing 50 ppm [^{14}C]-narasin for five consecutive days up to the time of slaughter. Three males and two females, not selected for the treatment group, were maintained on grower broiler ration and slaughtered at the same time as the treated chickens. The tissues from these chickens were used as a source of control tissue.

Excreta Collection. Excreta were collected from each of the treated pens beginning one day before the start of [^{14}C]-narasin feed dosing and continuing until the end of treatment. Excreta were stored frozen at −20 °C until processed.

Tissue Collection and Preparation. Chickens were sacrificed by electric stunning knife on day 5. At slaughter, samples of thigh and breast muscle (composite), abdominal fat, skin with attached subcutaneous fat, bile, the entire liver, and both kidneys were collected. The tissues were washed with water when collected and then frozen. Muscle, fat, and skin/fat were partially thawed and then homogenized in a food grinder. Livers and kidneys were minced with a scalpel while partially frozen. Tissues were then stored frozen at −20 °C until analysis.

Radiochemical Analysis of Tissues. Five 0.5 g replicates of muscle, skin/fat, liver, and kidney from each treated chicken were digested in 3 mL of Soluene 350 (Packard Instrument Co., Downers Grove, IL) for several days. When the tissues were completely digested, toluene-based scintillation cocktail was added for LSC. Five 1 g replicates of fat were melted at

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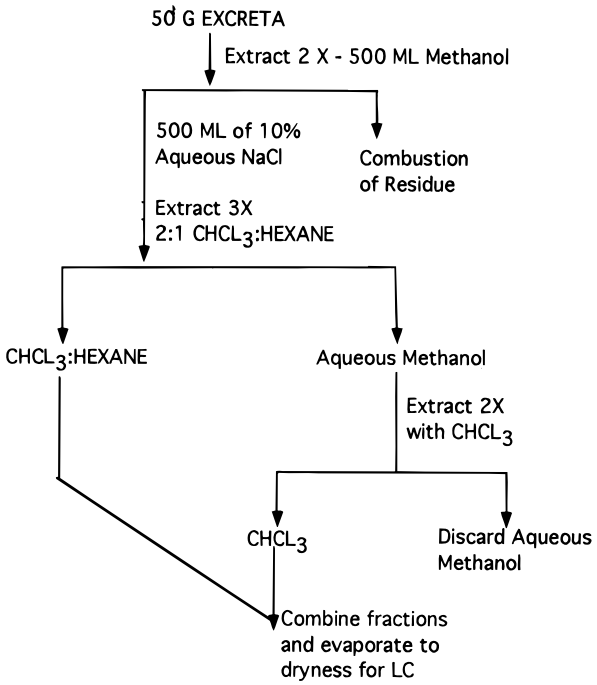

Figure 2. Flow chart for the isolation of metabolites from chicken excreta.

70 °C in counting vials and combined with Aquasol (New England Nuclear Corp., Boston, MA). Tissue samples from control chickens were used as background samples for LSC. All solubilized tissue samples were counted for a minimum of 10 min in a Tri-Carb liquid scintillation counter (Packard, Meriden, CT). Counting efficiency for tissue samples was determined by internal standardization with [^{14}C]toluene and count rates were adjusted to 100% efficiency.

Recovery of Radioactivity in Tissues. Recovery samples were prepared for muscle, skin, liver, kidney, and fat by adding 33 dpm of [^{14}C]narasin in 200 μL of methanol to aliquots of control tissues. The recovery samples had concentrations representing tissue concentrations of 0.067 ppm for muscle, skin/fat, liver, and kidney and 0.034 ppm for fat.

Characterization of Residues in Excreta and Tissues. Excreta, collected on the fifth day of dosing, containing equal amounts of excreta from each treated chicken was blended 1:1 with water. A 50 g aliquot of the blended excreta was extracted twice with 500 mL of methanol. Each methanol extract was filtered using Hy-flo as a filter aid. After the second filtration, the filter cake was dried, weighed, and combusted using a Model OX500 Biological Oxidizer (Harvey Instrument Corp., Hillsdale, NJ), and radioactivity was counted using LSC. The two methanol extracts were combined, and 500 mL of 10% aqueous sodium chloride solution was added to the extract. The methanolic solution was extracted once with 500 mL of 2:1 chloroform/hexane and twice with 250 mL of 2:1 chloroform/hexane. The methanolic solution was then extracted once with 500 mL and once with 250 mL of chloroform. The 2:1 chloroform/hexane and chloroform phases were combined and evaporated to dryness for preparative silica LC (Figure 2).

Pooled liver was extracted in a similar manner to that of excreta. A 50 g sample of pooled liver from the treated chickens was homogenized with 100 mL of methanol, shaken on a shaker table for 30 min at 200 rpm, and centrifuged until the supernate was free of solid material. The extraction procedure was repeated three times. The precipitate was dried, weighed, and combusted using a Model OX500 Biological Oxidizer and radioactivity was counted using LSC. The supernates were combined in a beaker and evaporated to 200 mL, and 100 mL of 10% (w/v) aqueous sodium chloride solution was added. The mixture was then extracted three times with 100 mL of chloroform. The chloroform fractions were combined and evaporated to dryness. The aqueous methanol solution

Table 1. ^{14}C Radioactive Residues as Narasin Equivalents (ppm) in the Primary Edible Tissues of Chickens Fed 50 ppm [^{14}C]Narasin Rations[a]

bird no.	sex	liver	kidney	muscle	fat	skin/fat
1	M	0.421	0.046	0.014	0.106	0.055
2	M	0.374	0.030	NDR	0.088	0.082
3	M	0.308	0.050	0.012	0.104	0.053
4	F	0.247	0.043	0.044	0.103	0.052
5	F	0.247	0.043	0.053	0.181	0.163
	mean	0.319	0.042	<0.04	0.116	0.081
	SD	0.077	0.008	0.021	0.037	0.048

[a] NDR = no detectable residue. SD = standard deviation.

was extracted three times with 100 mL of hexane. The hexane fractions were combined and extracted four times with 120 mL of acetonitrile. The combined acetonitrile fractions were evaporated to dryness and with the combined chloroform fractions were resuspended in 200 mL of 80/20 methanol/10% aqueous sodium chloride. An additional 56 mL portion of aqueous sodium chloride was added to this solution. The aqueous/methanol solution was then extracted three times with 125 mL of 2:1 chloroform/hexane and three times with 125 mL of chloroform. The organic fractions were combined and evaporated to dryness for preparative silica LC.

The evaporated excreta and liver extracts were resuspended in 5−10 mL of chloroform, evaporated onto 5−10 mL of silica gel which was placed at the head of a 60 cm × 1 cm i.d. column packed with Adsorbentein silica gel (particle size 50-200 μm; ICN, Irvine, CA). Radioactivity was eluted using a nonlinear gradient developed by pumping solvent from a 190 mL stirred reservoir filled initially with toluene. The volumes and solvent sequence were as follows: 40 mL toluene, 400 mL toluene/ethyl acetate 1:1, 400 mL ethyl acetate, 400 mL ethyl acetate/methanol/water, 98:2:0:05, 400 mL ethyl acetate/methanol/water, 90:10:1, 400 mL ethyl acetate/methanol/water, 80:20:2, 400 mL ethyl acetate/methanol/water, 60:40:4, 200 mL methanol. Twenty milliliter fractions were collected from the column, except for the final 200 mL of methanol which was collected in bulk. Following the methanol elution, the silica gel packing was removed from the column, dried, and assayed for radioactivity by combustion to determine if the radioactivity had been completely eluted from the column. Aliquots from each eluted column fraction were counted by LSC. In the case of excreta, selected fractions were combined for high-performance liquid chromatography/electrospray-mass spectrometry/liquid scintillation counting (HPLC/ESMS/LSC).

A 20 g sample of fat (pooled from the five chickens) was combined with 100 mL of hexane and melted at 70 °C. The hexane was decanted, and the precipitate was extracted again with 100 mL of hexane. The hexane was again decanted. The combined hexane extracts were extracted four times with 200 mL of acetonitrile. The acetonitrile was evaporated to dryness and the residue was resuspended in 10 mL of chloroform. The chloroform was passed through an unconditioned silica Sep Pak (Waters, Milford, MA), and the retained radioactivity was eluted with 10 mL of methanol. The methanol was evaporated to dryness, and the residue was suspended in 100 μL of methanol for HPLC/ESMS/LC.

HPLC and Mass Spectrometry. The metabolite pattern of radioactivity was determined in sample extracts by HPLC/ESMS/LSC. HPLC was performed using a 600-MS high-performance liquid chromatography solvent delivery system equipped with a Model 717 autosampler (Millipore Corp., Milford, MA). Mass spectrometry was accomplished using a PE-Sciex API-I (PE Sciex, Toronto, Canada) equipped with an electrospray interface. The effluent from the HPLC was split 19:1 where 50 μL/min were nebulized at the electrospray interface for MS and 950 μL/min were collected for LSC counting. Parent narasin in excreta was separated using a Zorbax SB-C8 column (5 μm, 4.6 mm × 25 cm) (Rockland Technologies, Inc., Newport, DE). The mobile phase was solution A, 10 mM ammonium acetate in water/acetonitrile (95:5); and solution B, acetonitrile/water (99:1). A linear

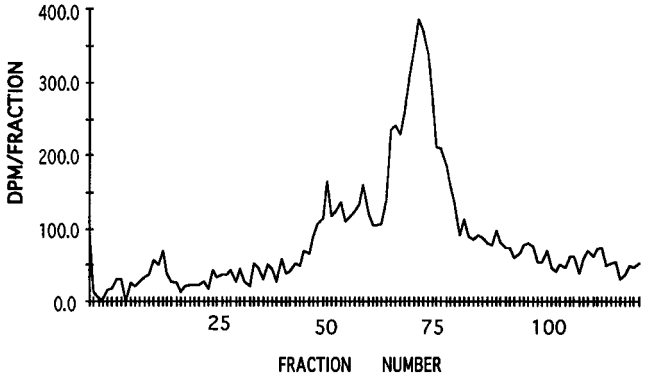

Figure 3. Radiochromatogram of a pooled liver extract constructed by liquid scintillation counting of fractions collected from a preparative silica column.

gradient was run from 50% A and 50% B at 0 min to 100% B at 18 min. Fractions for LSC were collected every 0.33 min.

Narasin metabolites in excreta were separated using the same column as for parent narasin in excreta. The mobile phase for separation was solution A, 2 mM ammonium acetate in water/acetonitrile (95:5); and solution B, acetonitrile. A linear gradient was run from 80% A and 20% B at 0 min to 50% A and 50% B at 50 min. Fractions for LSC were collected every 0.50 min.

Narasin and its metabolites are ionophores and thus will readily complex with cations. By using a small amount of sodium chloride in the sample and ammonium acetate in the mobile phase, both the sodium and ammonium adducts of narasin metabolites were observed. This aided in the identification of the metabolites by giving characteristic mass spectrometric doublets differing by 5 u between the ammonium and sodium adduct (Stout *et al*., 1991).

Parent narasin in fat was separated using a Jones C-18 column (5 μm, 4.6 mm $\times$ 25 cm) (Jones, Denver, CO). The mobile phase was solution A, methanol/water (95:5); and solution B, water. A stepwise linear gradient was run from 70% A and 30% B at 0 min to 85% A and 15% B at 4 min, to 100% A at 15 min. Fractions for LSC were collected every 0.33 min.

Thin-Layer Chromatography (TLC). For comparison of metabolites in excreta, a 20 $\times$ 20 cm plate of LK5 (Whatman, Maidstone, England) with a solvent system of ethyl acetate/acetone/ammonium hydroxide 100:100:5 was used. Extracted excreta samples were compared against ^{14}C standards of known metabolites which had been previously isolated from chicken excreta.

RESULTS

Liver was the tissue with the highest residue levels at slaughter. The mean liver concentration was 0.32 ppm of narasin equivalents for the pooled liver. Fat had the next highest concentration of residues of 0.12 ppm. All other collected tissues had residues of 0.08 ppm or less (Table 1).

Pooled liver was subjected to liquid/liquid extraction and silica column chromatography with counting of the silica column fractions. A radiochromatogram was constructed from the LSC counts (Figure 3). Because of the low concentrations and wide distribution of radioactivity in the liver fractions, no further characterization of liver metabolites was possible.

Fat was dissolved in hexane and extracted with acetonitrile. Ninety percent of the radioactivity was extracted from hexane. After purification on a silica Sep-Pak the sample was analyzed by HPLC/ESMS/LSC. The effluent from the HPLC was split 19:1 where 50 μL/min were nebulized at the ion spray interface for MS and the remaining 950 μL/min were collected for LSC counting. This procedure allowed for the simultaneous

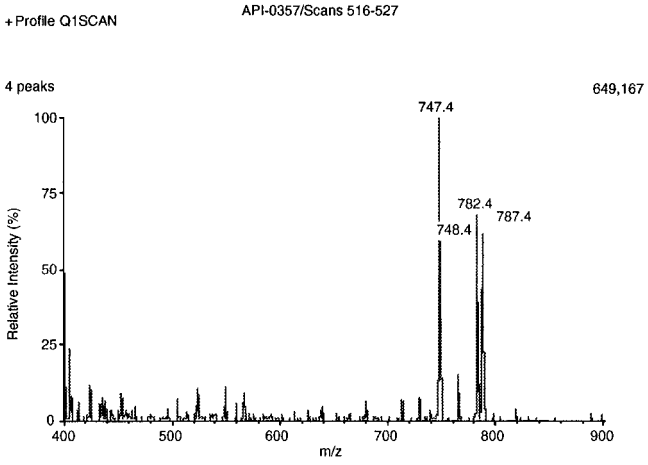

Figure 4. Mass spectrum of the pooled fat sample.

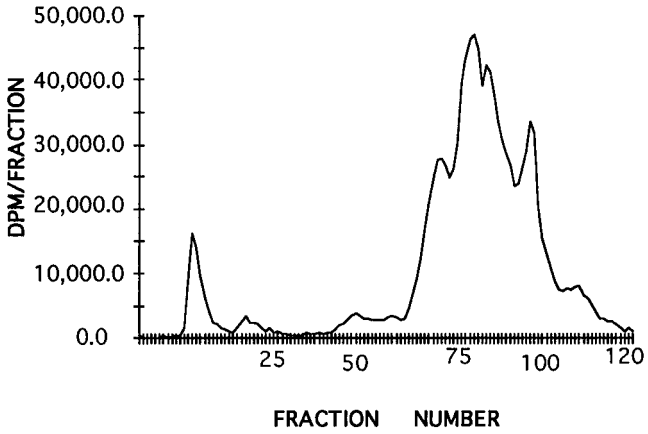

Figure 5. Radiochromatogram of an excreta sample. Radioactivity was isolated by liquid−liquid extractions and then further purified by elution from a preparative silica column.

construction of a radiochromatogram and total ion chromatogram (TIC). The radiochromatogram from fat had one predominant peak with a retention time identical to that of a narasin standard. The mass spectrum of the peak showed an ammoniated ion at *m*/*z* 782 and a sodiated ion of *m*/*z* 787 (Figure 4) consistent with the mass spectrum of a narasin standard. Parent narasin represented 61% of the total radioactivity in fat.

The radiochromatogram from a pooled excreta sample which had been extracted and fractionated on a preparative silica column is shown in Figure 5. Fractions 13−18 and fractions 67−103 were combined into respective pools and concentrated, and the two pools were individually run on HPLC/ESMS/LSC. The radiochromatogram had a predominant peak with an ion of *m*/*z* 782 and is consistent with the molecular weight of ammoniated narasin. Narasin appears on the mass spectrum exclusively as the ammonium adduct because of the addition of ammonium ion in the LC mobile phase. This peak accounts for 3.0% of the total radioactivity in excreta and has the same retention time as a narasin standard. Thus, the identity of the primary component in the peak from fractions 13 to 18 from the preparative silica gel column was narasin. Another smaller peak which eluted just after the narasin peak had an ion of *m*/*z* 796 and has a molecular weight that is consistent with a methylated narasin ammonium adduct. This peak represented less than 0.25% of the total radioactivity in the excreta.

Fractions 67−103 from the preparative silica column (Figure 5) were pooled and run by HPLC/ESMS/LSC.

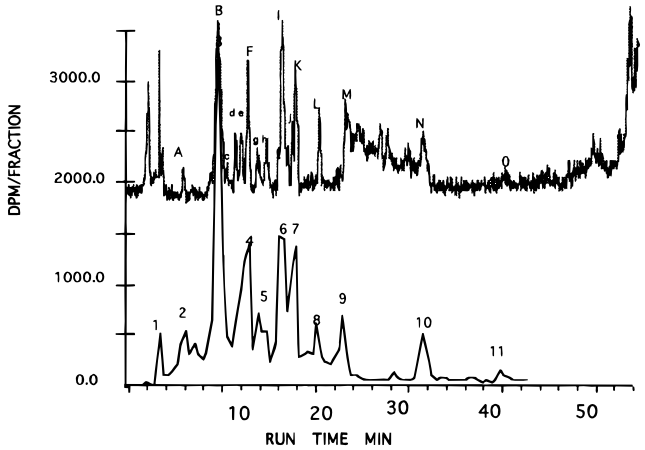

Figure 6. TIC superimposed on radiochromatogram of narasin metabolites extracted from excreta. Radiochromatogram was constructed by collecting fractions from HPLC/ESP-MS and counting the radioactivity on a liquid scintillation counter.

A TIC superimposed on a radiochromatogram is presented in Figure 6. Eleven peaks numbered 1 through 11 were identified by fraction collection and liquid scintillation counting as being radioactive and thus narasin-derived peaks. Fifteen peaks, lettered A through O, were identified as having the characteristic ion doublet. Table 2 shows the ion doublets and the percent total radioactivity for the excreta metabolites, as well as a proposed identity for each peak. Figure 7 shows selected mass spectra for the metabolites. An attempt was made to induce fragmentation using collision-induced disassociation (CID) by varying the orifice voltage. However, because of the low concentrations and matrix effects, structural information as to the position(s) of ring hydroxylation for the metabolites could not be determined. Fifteen narasin metabolites were characterized in chicken excreta. Five of these metabolites were hydroxylated metabolites of narasin, and the other 10 with masses 2 u less than the narasin-hydroxylated metabolites were presumed to be corresponding metabolites of narasin factor B, 20,20-didehydronarasin, which is narasin with the hydroxyl group on ring C oxidized to the ketone (Occolowitz *et al*., 1976). These 15 metabolites accounted for almost 50% of the total radioactivity in excreta.

In a previous study at our facility, Manthey and Goebel (1982) isolated and identified six narasin metabolites from chicken excreta which were characterized by electron impact mass spectra. They were able by fragmentation to assign the position of ring hydroxylation to six metabolites. These were designated NM1, NM2, NM3, NM4, NM6, and NM7. NM2 and NM7 were narasin which was trihydroxylated on rings A, B, and E (see Figure 1). NM1 was identified as narasin hydroxylated on rings C and E. NM3 was narasin hydroxylated on rings A and B, and NM4 was narasin hydroxylated on rings A and E. NM6 was narasin hydroxylated on rings B and E. In their study, trihydroxynarasin, NM2, was the most abundant metabolite. Five of these metabolites had been isolated in sufficient quantity to compare by TLC against the excreta metabolite fraction from this study (Figure 8). Fractions 67−103 from the preparative silica column (nar-1) and a fraction obtained by the same procedure from chickens treated with narasin in combination with nicarbazin (nar-2) were compared against the five standards. A prominent spot with an R_f value of 0.30 was found in the excreta fractions which has an identical R_f value to NM2. The TLC data coupled with the MS data provides evidence that peak B and possibly peak C is NM2. In addition, there was a prominent spot in the excreta fraction with an identical R_f value for NM3 which has previously been identified as dihydroxynarasin. There was also radioactivity from the excreta sample corresponding to NM1 and NM6. There was little if any NM4 in the excreta sample. This may be because the sample extraction and purification scheme was different in the two studies.

From the excreta, approximately 4% was nonextractable with another 6% to 8% remaining in the aqueous methanol extraction solvent. The remaining 90% of the radioactivity was extracted into organic solvents and fractionated by LC. Selected fractions from the LC were combined for HPLC/ESMS/LSC. Almost 50% of the total radioactivity was characterized as hydroxylated metabolites and another 3% was identified as parent narasin. The remaining radioactivity was in minor fractions from the preparative LC column and was in concentrations too low to characterize by HPLC/ESMS/LSC.

DISCUSSION

Following the administration of 50 ppm narasin in feed for five days, liver had the highest residue level of

Table 2. Narasin Metabolites Characterized in Excreta: Peaks Determined from Overlay of TIC[a] on the Radiochromatogram

TIC peak	radiochromatogram peak	ammoniated/sodiated adducts	% total radioactivity	proposed structure
	1		0.95	
A	2	846/851	2.44	tetrahydroxynarasin
B	3	830/835	13.44	trihydroxynarasin
C	3	830/835	*b*	trihydroxynarasin
D	4	828/833	6.94	trihydroxynarasin B
E	4	828/833	*b*	trihydroxynarasin B
F	4	828/833	*b*	trihydroxynarasin B
G	5	828/833	3.04	trihydroxynarasin B
H	5	828/833	*b*	trihydroxynarasin B
I	6	812/817	6.18	dihydroxynarasin B
J	7	814/819	4.39	dihydroxynarasin
K	7	814/819	*b*	dihydroxynarasin
L	8	812/817	1.86	dihydroxynarasin B
M	9	814/819 812/817	1.88	dihydroxynarasin/dihydroxynarasin B
N	10	814/819 828/833	1.86	dihydroxynarasin/trihydroxynarasin B
O	11	828/833	0.48	trihydroxynarasin B
% of total excreta radioactivity			43.46	

[a] TIC is Total ion chromatogram. [b] The peak is cumulative with preceding peak for % radioactivity.

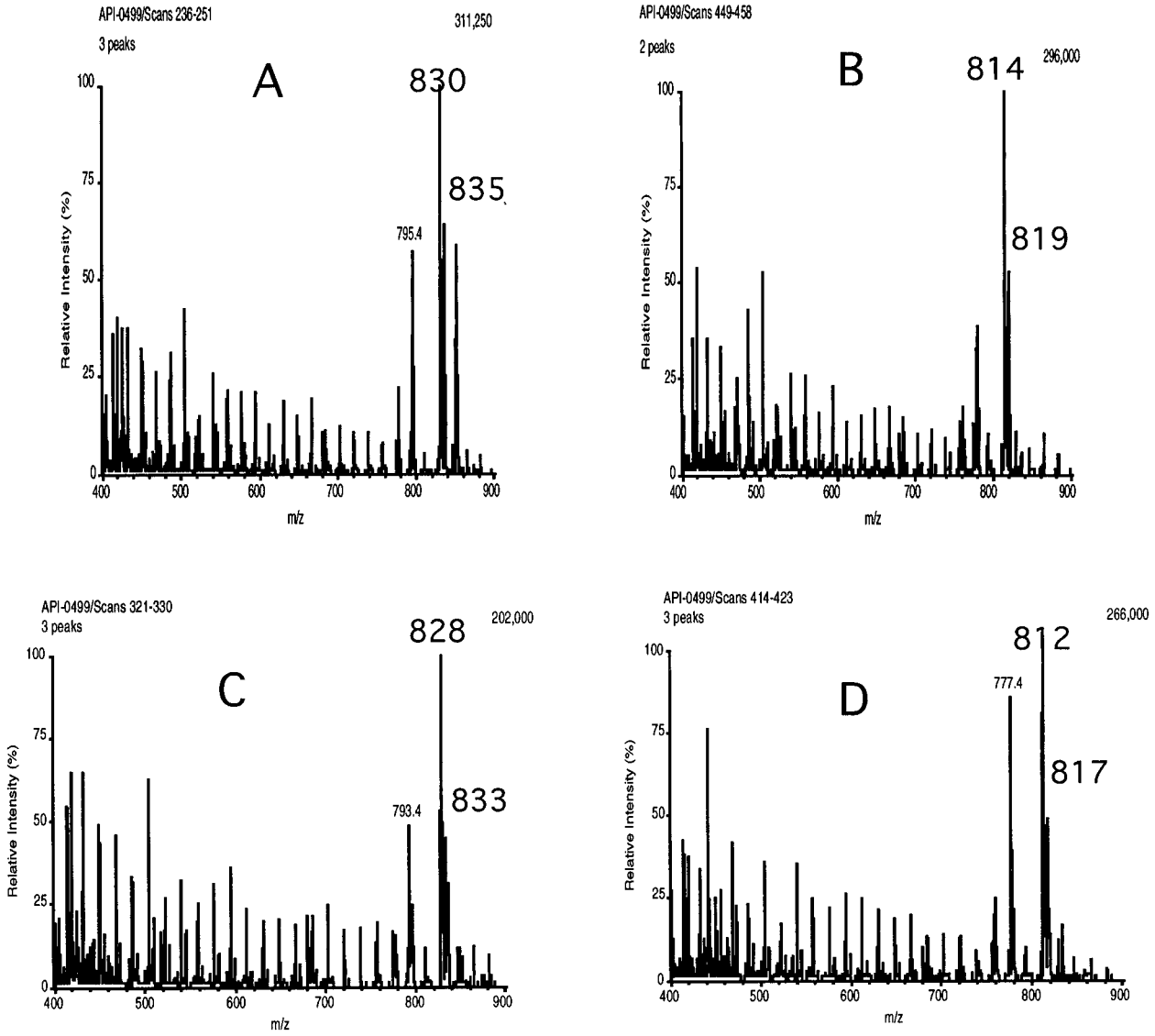

Figure 7. HPLC/ESP-MS mass spectra of selected metabolites in chicken excreta from Figure 7: (A) mass spectrum of peak B, trihydroxynarasin; (B) mass spectrum of peak K, dihydroxynarasin; (C) mass spectrum of peak F, trihydroxynarasin B; (D) mass spectrum of peak I, dihydroxynarasin B.

the tissues collected. Individual metabolites could not be identified because of the low amount and wide distribution of radioactivity in liver. However, the radiochromatogram from the liver (Figure 3) when compared to the radiochromatogram from the excreta (Figure 5) had similar profiles, suggesting that metabolites found in excreta might also be found in liver. In the liver radiochromatogram, there is an increase above background radioactivity at fractions 13−18 which suggest the presents of parent narasin in the liver. The bulk of the liver radioactivity eluted between fraction 60 and 85 where much of the hydroxylated excreta metabolites had eluted. Fat had the next highest residue level which was predominately parent narasin. No attempt was made to identify metabolites in the other collected tissues because of the low amount of residues. Dimenna *et al.* (1989) reported that chickens dosed for five days with 60 g/ton (55 ppm) salinomycin, a homologue of narasin differing by one less methylene group, had residues of 0.39 ppm in liver and 0.06 ppm in skin/fat which are similar to the values determined in this study. However, they also reported that chickens dosed at 75 g/ton (68 ppm) with salinomycin had residue values which were much higher than would have been anticipated from their previous study. Liver residue levels were close to the tolerance limit of 1.8 ppm and fat residue levels were at 0.2. Pretreating the chickens with salinomycin prior to dosing with [^{14}C]salinomycin, and use of a different dosing vehicle could not account for the differences in residue levels between their two studies.

Excreta samples were used as a source for metabolite characterization. Fifteen narasin metabolites were characterized. Five of these metabolites were hydroxylated metabolites of narasin and the other 10 were hydroxylated metabolites of narasin B. Narasin B, 20,20-didehydronarasin, is an oxidation product of narasin. A comparative metabolism study in rats done at our facility (Sweeney *et al.*, 1994) showed that fecal metabolites consisted of hydroxylated metabolites of narasin and narasin B indicating that the rats used for toxicology testing of narasin had been exposed to the same metabolites as were found in chicken.

While other ionophores, monensin (Donoho, 1984) and semduramicin (Lynch *et al.*, 1992) in chicken and maduramicin (Stout *et al.*, 1991) in turkey, undergo O-demethylation as the primary route of metabolism followed by hydroxylation, narasin has no methoxy

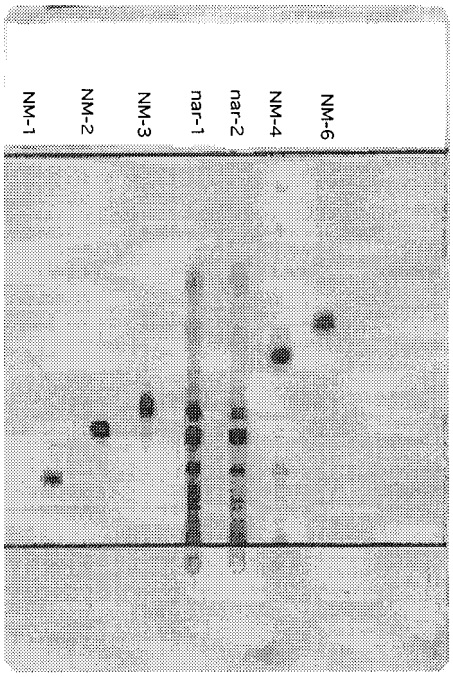

Figure 8. TLC autogradiogram of extracted excreta samples compared against previously isolated narasin chicken metabolites.

groups that can undergo O-demethylation; therefore, hydroxylation is the main metabolic pathway for narasin in chickens.

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