Analysis of Tissue Residues and Comparative Metabolism of Ardacin in Cattle, Broilers, and Rats

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Following continuous administration, ardacin residues equilibrated in tissues of cattle and broilers after 13 and 22 days, respectively, with average total residues of <111 ppb in all tissues. Kidney contained the highest residues in cattle, while liver was higher in broilers. Milk residues from treated dairy cows averaged only 2.1 ppb. Extraction of liver and kidney using 50% KOH/ACN removed 23–68% of the total radioactive residues. Balance-excretion studies demonstrated that ≥98% of the administered radioactivity was eliminated in the feces of all species studied. Comparative metabolism studies demonstrated that ardacin was not significantly biotransformed as the parent compound was detected in excreta and tissues from cattle, broilers, and rats. Microbiological assays of tissue and fecal extracts confirmed the presence of ardacin as antimicrobial potency results paralleled those obtained by HPLC. The low tissue residues and favorable safety profile has qualified ardacin for 0-day withdrawal in both cattle and broilers.

Keywords: *Ardacin; residues; metabolism; glycopeptides; food safety*

Ardacin is a glycopeptide antibiotic of the vancomycin family (Williams et al., 1980). The complex is produced by *Kibdelosporangium aridium* (ATCC 39323) and is composed of several closely related factors (Shearer et al., 1985; Sitrin et al., 1985). The ardacin structure is composed of a heptapeptide aglycon nucleus (derived biosynthetically from tyrosine, L-methionine, and sodium acetate), a D-mannosyl side chain, and an *N*-acyl lipid side chain. Differences in the ardacin factors are the result of slight modifications (additional methylene units, branching, unsaturation) in the lipid side chain (Jeffs et al., 1985, 1986).

Ardacin is defined for regulatory review and purposes of this discussion as containing 3 major factors (A, B, C), 6 minor factors (B2, B3, C1, C2, D, S), and a 10th component (HP-4), which results from hydrolysis of the *N*-acyl lipid side chain producing the mannosyl aglycon. HP-4 is thus produced in common from all other factors. The final ardacin product has a minimum chemical purity of ≥78% on an anhydrous basis and is thus not considered as a biomass. The remaining ≈20% of nonardacin components are byproducts of the fermentation and consist primarily of carbohydrate and lipid materials. The ardacin structure is shown in Figure 1.

Ardacin is under development as a growth promotant for beef cattle, dairy cows, and broilers, worldwide, and has recently received Annex II approval for broilers in Europe. A complete sequence of tissue residue and metabolism studies was conducted to satisfy the human food safety requirements for product registration. The results of these studies are the subject of this paper.

EXPERIMENTAL PROCEDURES

[^{14}C]Ardacin. Radiolabeled ardacin was prepared by fermentation using L-[U-^{14}C]tyrosine as the precursor. Biosynthetic studies (Chung et al., 1986a,b) have demonstrated that this procedure results in the incorporation of the label into a total of 42 carbon atoms within the aglycon ring as shown in Figure 1.

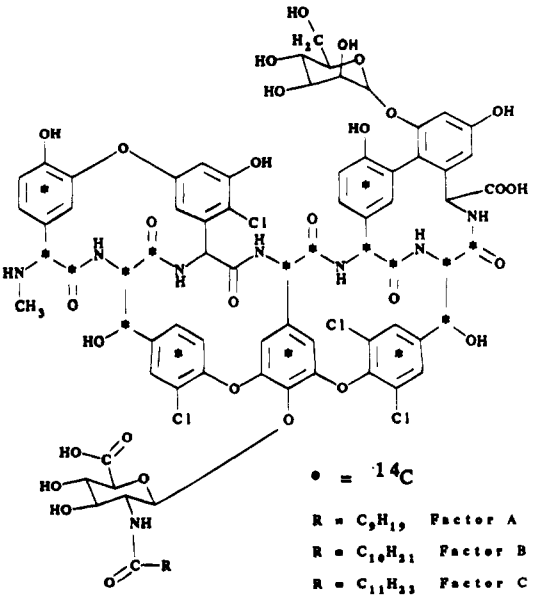

Figure 1. Structure of ardacin and ^{14}C-labeling pattern.

The crude fermentation broth was applied to an SP-207 column, washed with distilled water and 15% acetonitrile/water (ACN/H_2O) followed by 50% ACN/H_2O which eluted the ardacin product. Final material was obtained using preparative reversed phase gradient HPLC. The initial specific activity of approximately 5 mCi/g was appropriate for the rat comparative metabolism and broiler balance-excretion studies since only minimum gram quantities were required. For the beef cattle, dairy cow, and broiler equilibration studies, however, this material was diluted with appropriate amounts of nonradiolabeled ardacin to specific activities of approximately 1.0 mCi/g.

Final chemical purity was determined by HPLC (ACN/H_2O gradient) based on an ardacin reference standard. Since multiple components are included in the ardacin complex, purity was determined on the basis of total ardacin peak area in the sample. Radiopurity was determined by scintillation counting of collected fractions from an individual HPLC injection and by reversed phase TLC using a 0.1 M sodium

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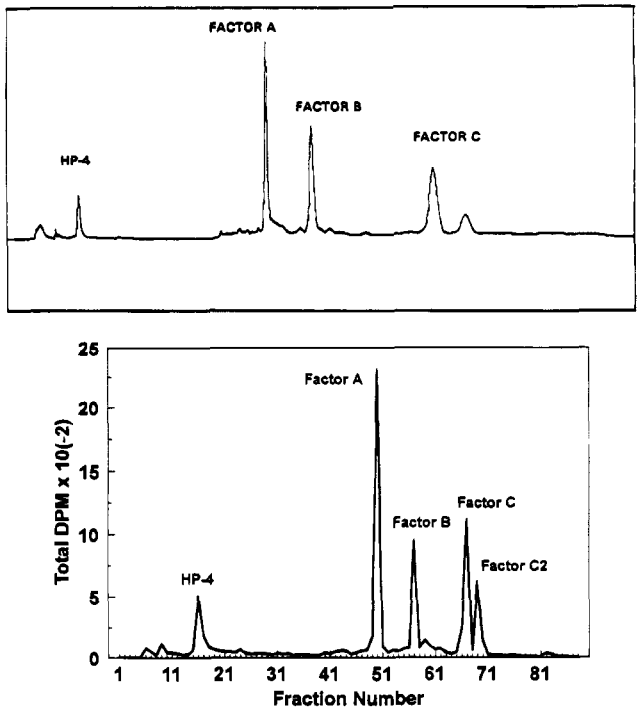

Figure 2. HPLC profiles: (a, top) UV trace of ardacin reference standard; (b, bottom) radiochromatogram of purified [^{14}C]ardacin following fermentation.

phosphate buffer (pH 7)/ACN in a 70:30 ratio followed by Bioscan analysis. In all cases, the final [^{14}C]ardacin assayed to radiochemical and anhydrous chemical purities of ≥98% and ≥78%, respectively. The water content (Karl-Fisher) of the final [^{14}C]ardacin batches was approximately 13%, to give an "as-is" chemical purity of 68–71%. All tissue residue calculations were based on a specific activity corrected for the final "as-is" chemical purity. Microbiological potency was determined using an agar well assay (see below). These values (≥78%) correlated well with the chemical purity established by HPLC. Representative HPLC chromatograms for the ardacin reference standard and [^{14}C]ardacin test material are shown in Figure 2.

Formulation. The [^{14}C]ardacin was prepared as a 26.4% premix with a rice hulls/mineral oil (1%) carrier. This premix was used as a top-dressing for dosing of beef cattle in the tissue residue study. Because of different requirements for the European market, a 25% $CaCO_3$ premix was used for medicated feed preparation at 15 and 50 g/ton for the broiler residue studies.

Animals. Broiler chickens (White Mountain Cross) were purchased from Martin's Hatchery, housed five to seven per cage, and given free access to unmedicated feed and water. After 1 week, the birds were housed individually to allow for monitoring of feed intake, and acclimation continued for an additional 2 weeks. The room temperature was kept at 88 °F for 10 days and progressively lowered 5 °F each week until a maintenance level of 65 °F was reached. Humidity was maintained at 37–60% throughout the study.

Hereford cattle (≈1 year old) and Holstein dairy cows (≈3–6 years old) were obtained from Thomas H. Geyer, Harleysville, PA. The animals were given a soybean supplement twice daily followed by corn silage. The animals were acclimated in a barn area for 1–4 months and then transferred to metabolism cages in a climate-controlled room (temperature and humidity at 60–80 °F and 25–55%, respectively). Electric milking machines were used for the Holsteins throughout the acclimation, medication, and withdrawal periods at approximately 12-h increments.

Sprague-Dawley rats were received from Charles River Breeding Laboratories, given free access to food and water, and acclimated for a period of 7 days in individual metabolism cages.

Residue Equilibration and Depletion Studies. *Poultry.* Six groups (four males, four females) were administered [^{14}C]-ardacin continuously in feed at a dose level of 15 g/ton (1.5–2.0 times the maximum anticipated commercial dose) for 10, 14, 18, 22, 26, or 30 days followed by sacrifice at 0 days of withdrawal (6 h after removal of medicated ration). An additional three groups (four males, four females) were administered feed at the same dose level for 30 days and subjected to withdrawal periods of 2, 4, or 7 days. Excreta (urine and feces were not separated) were collected from the 7-day withdrawal group at 48-h intervals and pooled by sex for each time point. A final group (six males, six females) was dosed at 50 g/ton (10×) for 30 days followed by a 7-day withdrawal period. A larger number of birds were included in this group to allow for mortality because of the increased medication/withdrawal time.

All birds were sacrificed via cervical dislocation at the appropriate time points. Immediately after sacrifice, the entire liver, both kidneys, and representative muscle and skin/fat samples were removed, frozen, and stored (−20 °C) until assayed. Tissue samples (0.3–1.0 g; five replicates/sample from all individual birds) were analyzed for total radioactivity content by combustion (Packard Oxidizer Model 307) followed by liquid scintillation counting (LSC). The final results, expressed as [^{14}C]ardacin equivalents, were averaged (males and females combined) and reported in parts per billion at each time point. Excreta samples were combusted similarly (0.5 g; five replicates of pooled samples) and the results expressed as a percent of the administered dose (calculated on the basis of feed intake) over the 48-h collection interval. Additional samples (15 g/ton from medication days 22–30, 0-day withdrawal, and 50 g/ton, 7-day withdrawal) were used for the generation of tissue and/or excreta metabolic profiles. Tissue samples from individual birds were pooled by sex prior to extraction for these experiments.

Cattle. After about 2 weeks in the metabolism cages, the cattle received a 25% [^{14}C]ardacin premix (split into two doses, 8 a.m. and 4 p.m.) top-dressed onto the soybean supplement for 10 (one male), 13 (one male, two females), 16 (two males, one female), or 20 (two males, one female) days at a dose equivalent to 45 g/ton in feed. Urine and feces were collected from the cattle treated for 16 days (one male, one female) at 12-h intervals during days 14–16 of the medication period for use in metabolic profiling. All cattle were sacrificed 10 h after the final dose (0-day withdrawal) by captive bolt. The entire liver, both kidneys, and representative muscle, renal fat, and subcutaneous fat were excised, frozen, and stored (−20 °C) until assayed. Tissue samples (0.3–1.0 g; five replicates per sample from individual cattle) were assayed for total radioactivity content using combustion followed by LSC. Urine aliquots were counted via LSC. Additional tissue samples collected from the cattle treated for 16 days (one male, one female) were used for the generation of the metabolic profiles. The male and female samples were profiled separately.

Dairy Cows. [^{14}C]Ardacin-containing gelatin capsules were administered to three dairy cows via an oral capsule gun followed by a small amount of feed to ensure complete consumption of the dosing material. The animals received 300 mg/head daily split into two doses (following morning and evening milking) for 10 consecutive days followed by a 6.5-day withdrawal period. Triplicate (3 or 5 mL) milk aliquots from individual animals after each 12-h collection interval were counted for total radioactivity content. The results from the three animals were averaged and reported in parts per billion for each collection period. Blood samples were collected at 0, 2, 4, and 8 h following the initial dose and immediately following each subsequent milk collection during the medication period. Triplicate aliquots (plasma) were counted for total radioactivity content. Sacrifice was by Beuthanasia overdose. No tissues were taken for this study.

Balance-Excretion Studies. *Poultry.* From 21 to 43 days of age, all birds received nonradiolabeled ardacin medicated feed at a level of 15 g/ton until equilibration at 22 days (*Note: this pre-equilibration protocol was requested by European regulatory agencies.)* Ten (five males, five females) broilers received a single oral dose of [^{14}C]ardacin premix via capsule

at a concentration of 3.5 mg/kg of body weight on day 44. All birds, including controls (three males, three females), received unmedicated grower feed until sacrifice. Excreta were collected at approximately 12-h increments and pooled by sex for 7 days following [^{14}C]ardacin administration. Excreta radioactivity was determined at each time point utilizing a combination of combustion and LSC techniques, and the results are reported as a percentage of administered dose (average of males and females combined).

Cattle. [^{14}C]Ardacin premix (25%) equivalent to 45 g/ton was administered to three cattle (one male, two females) in a gelatin capsule by dosing gun to ensure immediate consumption. Urine and feces were collected from each animal at 12-h intervals for 156 h. The heifers were catheterized, while the steer was fitted with a flexible hand-crafted funnel device (Veenhuizen et al., 1984) to ensure separate urine collection. Urine was assayed directly by LSC, and feces were assayed by a combination of combustion and LSC. The results from the three animals were averaged and reported as a percentage of administered dose for each collection period. Sacrifice was by captive bolt.

Rat Metabolism. Ten rats were randomly assigned (on the basis of body weight distribution) to group I, which received a daily 25 mg/kg oral dose of [^{14}C]ardacin as an aqueous solution for 14 days. An additional 10 rats comprised group II (controls), which received an equivalent volume of water for the same duration. Individual urine and feces samples were collected at 12-h intervals during the entire 14-day medication period. Samples from days 10−13 were subsequently pooled by sex on each day for analysis of total radioactivity, and the results are reported as a percentage of the daily administered dose.

The rats were sacrificed by asphyxiation in a CO_2 chamber 6 h after the final dose. Liver and kidney samples were pooled by sex and assayed for total radioactivity content by combustion and LSC, and the results are reported in parts per billion based on [^{14}C]ardacin equivalents.

Sample Extractions. *Urine.* Cattle and rat urine were adjusted to pH 6.0 with 1 N HCl, and 10 mL was applied to a 5 g/20 mL C_{18} Bond-Elute (Analytichem International) solid phase extraction column. The cartridges were washed with 10 mL of 0.02 M potassium phosphate buffer (pH 6.0) followed by consecutive elution with 10-mL portions of Et_2O, 10% ACN/H_2O, 50% ACN/H_2O, and 12 mL of 50% MeOH/H_2O. Aliquots were taken from each elution step and counted for radioactivity. The Et_2O and ACN/H_2O fractions were dried (25 °C) for HPLC analysis in a nitrogen stream.

Feces. Approximately 10-g samples (all species) were homogenized in 100 mL of 0.1 M KOH/ACN (50/50 v/v) followed by immediate adjustment to pH 8.0 with 5 M HCl. The samples were centrifuged at 2000−5000 rpm for 15 min, the liquid was decanted, and the pH was readjusted to 8.0, if necessary. The rat and broiler extracts (50 mL) were diluted with 100 and 50 mL of pH 8 potassium phosphate buffer (0.1 M), respectively, and applied (10 mL) to strong anion exchange columns (1 g/6 mL, Bond-Elute) preconditioned with 5 mL of MeOH and 5 mL of buffer [33% [0.1 M KOH/ACN (50/50)]/0.1 M potassium phosphate buffer, (pH 8, v/v]. The columns were washed with 5 mL of buffer followed by elution (2 × 5 mL) with 0.1 M HCl/0.2 M NaCl and MeOH (2 × 5 mL). The acid fractions were adjusted with Na_2CO_3 to approximately pH 7, and both eluents were dried for HPLC and microbiological analysis.

Tissues. Liver and kidney samples (50 g for cattle and broilers; 5 g for rat) were extracted with 3 × 50 mL (75 mL, broilers) MeOH followed by 3 × 75 mL of Et_2O. Centrifugation between extractions was performed at 2000−7000 rpm for 15 min. The supernatants were decanted and discarded. The remaining tissue pellet was extracted with 3 × 75 mL of 0.1 M KOH/ACN (50/50 v/v, centrifugation as above), and the resulting supernatants were combined, adjusted to pH 8, and reduced to a small volume on a rotary evaporator (≈35 °C) to remove the majority of the ACN. Aliquots (20 mL) of the concentrates were applied to a 5 g/20 mL C_{18} Bond Elute column (Analytichem) preconditioned with 5 mL of MeOH and 5 mL of buffer (0.02 M potassium phosphate, pH 6.0). The

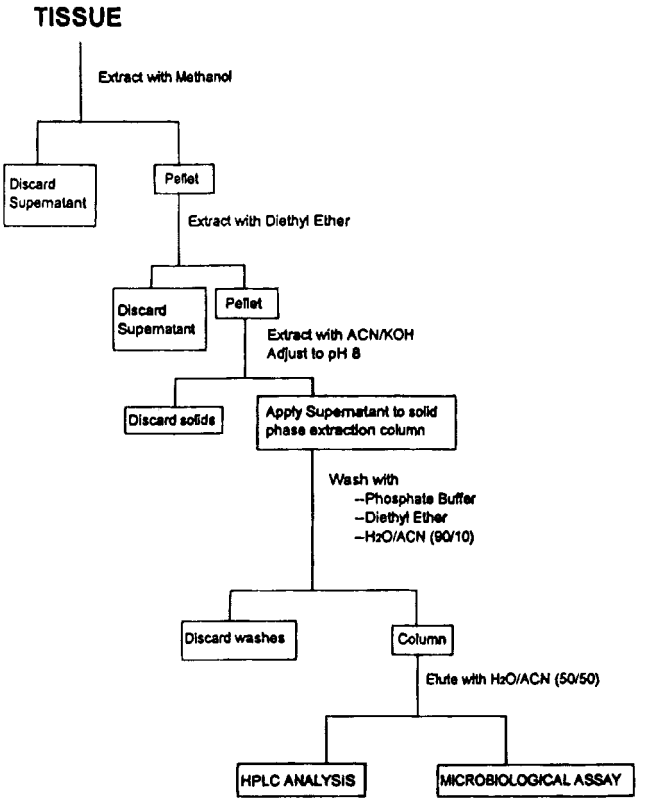

Figure 3. Extraction and sample preparation procedure for the liver and kidney tissues from cattle, broilers, and rats.

cartridges were washed with 10−15 mL of buffer followed by sequential elution with 10−15 mL of Et_2O, 2 × 10 mL of 10% ACN/H_2O (2.5% broilers), 2 × 10−20 mL of 50% ACN/H_2O, and 2 × 10 mL of 50% MeOH/H_2O. The 50% ACN/H_2O eluents were evaporated to dryness (N-Evap, 25 °C) for HPLC analysis. The extraction and cleanup procedure for the tissue samples is outlined in Figure 3.

HPLC. Chromatography was performed for cattle and rats (broilers) using the following equipment: dual Beckman Model 100A (Altex Model 110A) solvent delivery pumps, a Beckman Model 421 (Altex Model 420) controller, and a Waters Model 481 UV detector operating at 220 nm.

The cattle and rat extracts were analyzed on a Phenomenex-Ultrex C_{18} column, 25 cm × 4.6 mm, with a flow rate of 2 mL/min. The program was as follows: 0−2-min hold at 22% ACN/78% 0.02 M phosphate buffer, pH 6.0 (buffer); linear gradient over 3 min to 28% ACN/buffer; linear gradient over 22 min to 35% ACN/buffer; return to 22% ACN/buffer over 0.5 min; and re-equilibrate for at least 15 min prior to the next injection.

The broiler extracts were analyzed using a Supelcosil LC-18 column, 25 cm × 4.6 mm, with a flow rate of 1.5 mL/min. The program was as follows: 0−2-min hold at 22% ACN/buffer; linear gradient over 10 min to 32.5% ACN/buffer; maintain at 32.5% ACN/buffer for 18 min; return to 22% ACN/buffer over 2 min; and re-equilibrate as above. (Note: the gradient program for the broiler analysis was different from that used for rats and cattle. While comparative studies are generally conducted using identical methods, the separation of the ardacin factors was so far superior using the broiler method that the change was justified.)

For generation of the radiochromatograms, fractions were collected every 15 s for the duration of the run (88−120 samples), followed by direct scintillation counting for 20 min in 10 mL of Ultima Gold cocktail.

Microbiological Assay. The microbiological activity of ardacin in excreta and tissue extracts (as well as for raw chemical purity determinations) was assayed using an agar well diffusion method. Initial extracts were evaporated to dryness and reconstituted in 20% EtOH/80% 0.2 M KH_2PO_4 (pH 6.5) (assay diluent). The volume of assay diluent was adjusted on the basis of the total radioactivity present in the

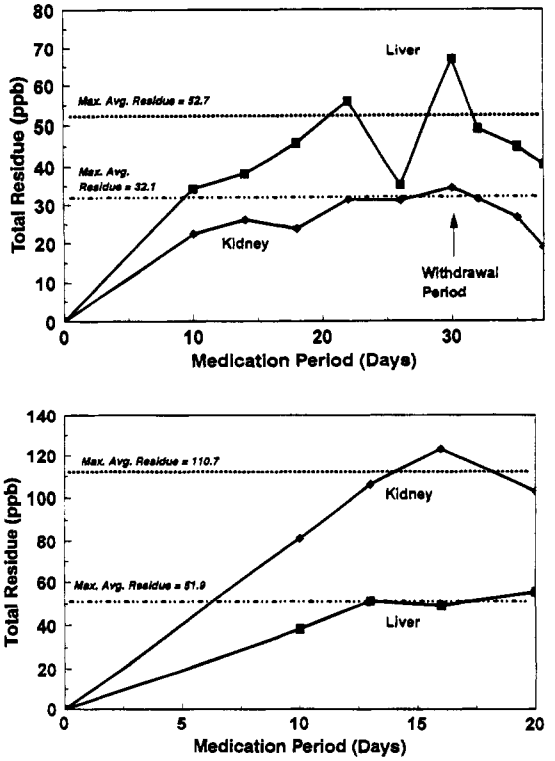

Figure 4. Equilibration of [^{14}C]ardacin in (a, top) broiler and (b, bottom) cattle tissues.

sample to maintain the ardacin concentration within the standard curve. Agar plates were seeded with *Corynebacterium xerosis* as the test organism, and samples were dispensed into the agar wells of three plates (per sample). The ardacin in the extracts diffused into the agar and produced zones of growth inhibition over a 15–22-h incubation period at 36 °C. Quantitation of ardacin was determined by comparison of the zone diameters from the extracts with those produced from a standard curve (0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 μg/mL ardacin reference standard in assay diluent) using log–linear regression analysis [zone size (mm) vs log [ardacin] (μg/mL)].

Statistical Analysis. Average and standard deviations (SD) of tissue/excreta residues were calculated on the basis of individual and/or pooled samples as described above.

Tissue equilibration time was evaluated using two methods. The mean, SD, and degrees of freedom were calculated for each set of data using the In-Stat statistics package from GraphPAD Software. A one-way ANOVA was performed to obtain the overall SD between groups. Comparisons were made between all group combinations by means of a t test, which generated a mean difference value for each individual combination. Statistical differences between groups were evaluated by calculating the least significant difference (lsd) value for the overall data set according to the formula

$$\text{lsd} = \sqrt{\frac{2[\text{SD}]^2}{n}}(t_{0.05})$$

The difference was considered significant if the absolute value of the mean difference was >lsd. Groups without significant differences were considered to be at equilibrium.

Additionally, selected groups determined to be at equilibration by the ANOVA method were further evaluated using linear regression. Slopes not significantly different from zero were considered to support equilibration.

RESULTS AND DISCUSSION

The tissue equilibration results for cattle and broilers are shown in Figure 4. For broilers (Figure 4a), total radioactive residues increased steadily until equilibration occurred after 22 days of continuous ardacin

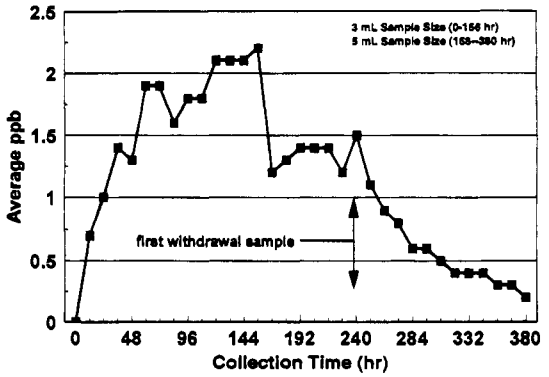

Figure 5. Equilibration and depletion of [^{14}C]ardacin in milk from dairy cows.

administration. Liver was the apparent target tissue since it contained the highest radioactivity, with maximum average residues of 52.7 ± 22.5 ppb. Kidney [collected and analyzed for European registration; not required by the Center of Veterinary Medicine (CVM)] levels averaged 32.1 ± 8.5 ppb, while skin/fat and muscle levels were below the limits of detection of ≈20 ppb. The determination of equilibration time was based on a statistical analysis of the residue data from all animals and defined as the point at which subsequent average values were not significantly different or when the slope of the regression line did not differ significantly from zero. While the broiler residues in liver were highly variable at days 22, 26, and 30, these data points met the above criteria for equilibration.

Total ardacin residues depleted slowly from broilers following equilibration (Figure 4a) as liver and kidney levels decreased to 40.3 ± 9.5 and 18.6 ± 9.7 ppb, respectively, after 7 days of withdrawal.

One additional group of birds, included to provide preliminary residue data for potential ardacin registration in Japan, was dosed at 50 g/ton (approximately 10 times the intended commercial dose) for 30 days followed by a 7-day withdrawal period. Higher total residue levels averaging 213.2 ± 81.9 and 73.1 ± 23.8 ppb were detected in the livers and kidneys, respectively, from these birds. Muscle and skin/fat samples remained below the limit of detection.

For cattle, equilibration occurred after 13 days of continuous ardacin administration (Figure 4b). In contrast to broilers, kidney was determined to be the target tissue followed by liver. The average total residues in these two tissues at equilibration were 110.7 ± 25.2 and 51.9 ± 13.8 ppb, respectively. Muscle and fat levels were extremely low and not quantifiable.

Milk collected from dairy cows orally treated with [^{14}C]ardacin at a daily dose level of 300 mg/head had detectable but very low levels of radioactive residues. Equilibration in the milk occurred after 5 days of continuous administration with an average concentration of 2.1 ppb (average SD ± 16.0% for all time points) total residues as shown in Figure 5. A change in sample volume from 3 to 5 mL for scintillation counting was made after 156 h in an attempt to increase sensitivity; however, this change resulted in the apparent decrease of total milk radioactivity when all samples were corrected for background and expressed on a parts per billion basis. This difference was attributed to experimental error due to the inherent difficulties in counting these low-level milk samples. The data have been interpreted as equilibration occurring at 120 h, and thus the radioactivity levels between 120 and 240 h are

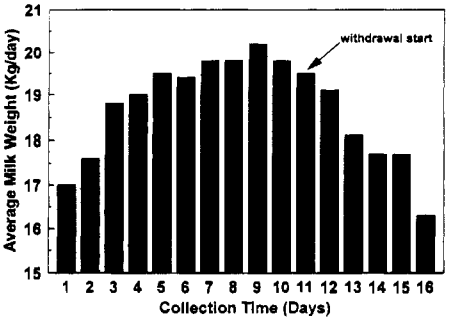

Figure 6. Total milk yield from dairy cows administered [^{14}C]-ardacin continuously via capsule at a daily dose level of 300 mg/head.

considered equivalent. Depletion was monitored using the 5-mL sample size starting at 240 h.

Following cessation of [^{14}C]ardacin administration, the milk radioactivity decreased gradually to near control levels (0.2 ppb) after 7 days of withdrawal. Plasma radioactivity in the dairy animals was extremely low, highly variable, and nonquantifiable in this study. For estimation purposes, an assumption was made that if 10 dpm (2 times the lowest validated level) was detected in a 3-mL aliquot, this would correspond to a total residue of 3 ppb on the basis of the specific activity of the [^{14}C]ardacin. The average plasma radioactivity levels were below 3 ppb at all times except the 24-h collection point.

While the primary purpose of the dairy cow study was residue determination, a secondary observation concerning total milk yield was of interest. During the medication phase, average milk production (Figure 6) increased from 17.0 to 20.2 kg/day, an increase of approximately 19%. This effect was apparent as early as day 2 of medication. Following withdrawal of the drug, milk production returned to premedication levels after only 6 days of withdrawal. Decreased milk weight was observed within 1 day of cessation of drug administration. Since the number of animals in the study was limited, no claims about the statistical significance of this result regarding product efficacy are made. Nevertheless, the observation was striking under these controlled conditions.

Total radioactive residues in the rat tissue samples were considerably higher, as expected, due to the use of the elevated daily dose of 25 mg/kg. Kidney was the target tissue in rats (similar to cattle), with total [^{14}C]-ardacin equivalent residues (pooled by sex) of 58.1 ppm in the males and 77.1 ppm in the females. Liver residues were lower at 15.3 and 31.8 ppm, respectively. All residue levels were determined after 6 h of withdrawal.

Results from the balance-excretion studies were consistent among all species and are summarized in Table 1. [^{14}C]Ardacin residues were excreted primarily in the feces, with less than 1% of the total collected radioactivity being found in the urine. Bile collected from beef cattle at sacrifice contained extremely low levels of radioactivity, indicating that elimination via this pathway is of no significance (data not shown). The fecal elimination profile comparison for broilers and cattle is shown in Figure 7. Excretion occurred rapidly, with the majority of the dose being recovered within 24 h for broilers and 48 h for cattle.

Because of the high water solubility of ardacin, extraction of radioactivity from tissues was effective only with aqueous or aqueous/organic solvent systems. For

Table 1. Excretion of [^{14}C]Ardacin Residues in Various Species

species	excretion percentage	
	urine	feces
cattle[a] (2M, 1F)	0.1 ± 0.0	87.8 ± 3.5
poultry[b] (5M, 5F)		73.4
poultry[c] (4M, 4F)		90.9 ± 14.6
rats[d] (5M, 5F)	1.1 ± 0.7	90.5 ± 33.4

[a] Average ± SD for three animals receiving a single dose of [^{14}C]ardacin premix equivalent to 45 g/ton in a gelatin capsule. [b] Data from the balance-excretion study in which broilers received a single oral dose of [^{14}C]ardacin premix equivalent to 3.5 mg/kg of body wt. Samples were pooled by sex at each time point prior to analysis. Value reported is the overall average of males and females. Urine and feces were not separated for poultry. Data are included in feces column for convenience and probable distribution based on comparison with other species. [c] Data from equilibrium study in which broilers received [^{14}C]ardacin in medicated feed at a level of 15 g/ton continuously for 30 days. Samples were pooled by sex at each time point. Average ± SD of percentage of daily dose excreted is reported for days 2–30. [d] Overall average (males and females) ± SD of percentage of daily dose excreted between days 10 and 13 when rats were orally administered [^{14}C]ardacin daily as an aqueous solution at a level of 25 mg/kg. Samples were pooled by sex at each time point.

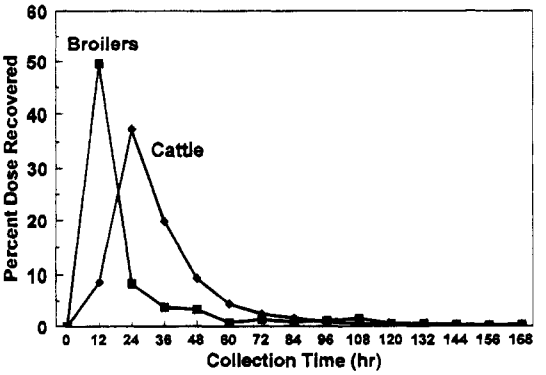

Figure 7. Elimination profile of [^{14}C]ardacin residues in the feces of beef cattle and broilers.

the metabolism studies, the highest recoveries were achieved using an extraction solution of 0.2 M KOH/ACN (50:50 v/v). While the basic conditions significantly enhanced the recoveries, rapid adjustment (within 30 min) of the initial extract to pH 6–8 was necessary to prevent ardacin hydrolytic degradation (to HP-4). Table 2 summarizes the extractability of [^{14}C]ardacin residues from liver and kidney tissues of broilers, cattle, and rats. Although KOH/ACN proved to be the best solvent system tested, extraction was still less than quantitative. Across species only about 34–65% of the total liver and kidney residues were extractable. Cattle tissues were the most difficult (≈34%), while rat kidney was the most readily extracted (≈65%). When [^{14}C]-ardacin standard was fortified into control tissues and processed via the identical extraction procedure, similar results were obtained with tissues having the incurred residues. Extractability in broilers decreased slightly as withdrawal time increased. Only 23.7% of the total tissue radioactivity was obtained from female liver after 7 days of withdrawal, while birds treated with [^{14}C]-ardacin at 50 g/ton showed slightly higher extractability (45.8% males, 48.5% females).

The slow depletion of tissue radioactivity and the decrease in extractability as withdrawal time increases are consistent with the binding of ardacin with tissue macromolecules (proteins), forming complexes that are not easily dissociated. This binding occurs rapidly, and

Table 2. Extraction of [^{14}C]Ardacin Residues from Tissues of Cattle Broilers and Rats

species	sample ID	extractable residues (%) liver	kidney
broilers,	fortified control	53.2	56.5
0-day withdrawal[a]	male	58.5	59.1
	female	43.2	47.7
broilers,	males	35.7	
7-day withdrawal[a]	females	23.7	
cattle,	fortified control	48.5	35.7
0-day withdrawal[b]	males	33.9	32.2
	females	38.3	30.6
rats,	fortified control	59.2	67.8
0-day withdrawal[c]	males	52.3	67.3
	females	46.2	62.7

[a] Tissues were pooled by sex (4M, 4F) prior to extraction. Data reported are from a single pooled sample following the method in Figure 3. [b] Data from 1M and 1F following treatment for 16 days with [^{14}C]ardacin premix. [c] Tissues were pooled by sex (5M, 5F) prior to extraction. Data reported are from a single pooled sample following the method in Figure 3.

depletion of these bound residues is likely to be controlled, at least in part, by normal protein catabolism and turnover as opposed to the rapid elimination of free parent drug and/or metabolites. An early purification technique (Folena-Wasserman et al., 1987) utilized this ardacin/protein interaction property effectively as the complex was found to bind to specific amino acid ligands attached to an affinity column support.

In contrast to tissues, ardacin residues in cattle, broiler, and rat feces were readily extractable with KOH/ACN. Total recoveries from feces of dosed animals were 93–98%, 89–94%, and 74–110%, respectively, for these species. Extraction of fortified control feces gave an average recovery of 95.1%.

Ardacin metabolism was investigated in urine, feces, and tissues using common extraction and chromatography procedures for these matrices across species. Each matrix for each species included an experiment with a control sample, [^{14}C]ardacin standard fortified control, along with samples from both male and female dosed animals.

Radioactivity in the initial feces extracts was found to bind tightly to strong anion exchange (SAX) cartridges, which provided an effective means of sample cleanup. Elution was performed sequentially with 0.1 N HCl and MeOH (which improved column recoveries), although these two fractions were sometimes combined (cattle and rat) prior to HPLC analysis. Between 93% and 100% of the applied radioactivity was eluted in the acid and MeOH fractions for all species.

The HPLC radiochromatograms for the cattle and rat feces extracts are shown in Figure 8 (broilers not shown). The results clearly indicate that the majority of the radioactivity in the feces of all species is ardacin itself. The total peak radioactivity associated with ardacin factors A, B, C + C_2, and HP-4 averaged 85.3%, 87.0%, and 65.6% for cattle, rats, and broilers, respectively. After an adjustment for SAX column recovery and extraction efficiency, the total fecal radioactivity attributed to intact ardacin was 80.5%, 72.5%, and 58.5%. Several smaller peaks (unidentified) are evident in the radiochromatograms which amounted to <5% of the total radioactivity present in the sample. The total ardacin content of the broiler feces was lower than that reported for cattle or rats due to a high percentage of the applied radioactivity associated with the background (≈20%) and not with additional metabolites.

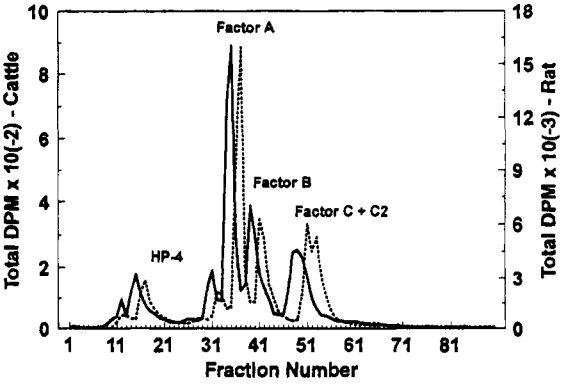

Figure 8. HPLC radiochromatogram of feces extracts from cattle (—) and rats (- - -). Note: the radiochromatogram for rat feces is offset by 30 s to allow profile comparisons.

Additional samples of the combined HCl and MeOH fractions from the SAX column cleanup procedure were analyzed for microbiological activity using an agar well method. The data were analyzed by assuming that 100% of the radioactivity in the sample was parent ardacin and thus active. For cattle, the results indicated that 98.3 ± 23.3% of the theoretical radioactivity in the extract was microbiologically active. For broilers, only 52.4% was active; however, this lower value was consistent for the broiler feces obtained from the dosed animals as well as from control feces fortified with the [^{14}C]ardacin standard. These results suggest that the lower recovery for broilers was either due to the analytical methodology or possibly due to unknown inhibitory factors present in the excreta which are not found in cattle feces (*Note: urine and feces were collected separately for cattle but not for broilers due to the inherent anatomical differences of the species.)* The close agreement between the microbiological activity values and the HPLC data supports the conclusion that the majority of the radioactivity is present as parent ardacin.

Urine contained ≤1% of the initial dose for both cattle and rats. Sample cleanup involved direct application of diluted urine samples to C_{18} cartridges followed by sequential elution with buffer, Et_2O, 10% ACN/H_2O, and 50% ACN/H_2O. For cattle, significant differences were seen in the elution profile for urine from the dosed animals vs the [^{14}C]ardacin standard fortified control. Over 73% of the applied radioactivity was eluted with the 50% ACN/H_2O fraction for the fortified control, while the dosed urine partitioned more evenly with 19.6%, 41.4%, 41.2%, and 17.3% in these four fractions, respectively. For rats, the profile for the dosed animals remained similar to the fortified control profile as the majority of the applied radioactivity was found in the ACN/H_2O fractions (64.6%), while <5% was present in the buffer and Et_2O fractions.

HPLC analysis was performed on both the Et_2O fraction (cattle only) and the combined (10% and 50%) ACN/H_2O fractions. The radiochromatogram of the cattle ether fraction (Figure 9) showed a single peak eluting with a retention time corresponding to the HP-4 hydrolysis product. In contrast, the radiochromatogram for the ACN/H_2O fractions (not shown) indicated the presence of the parent drug with all major factors represented (rat) along with a minor peak (unidentified) comprising 7% of the recovered counts. The corresponding fraction for cattle was more difficult to interpret due to low levels of total radioactivity in the sample. A significant amount of ardacin factor A was evident,

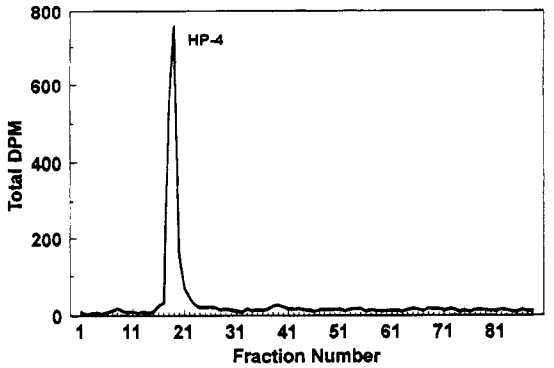

Figure 9. HPLC radiochromatogram comparison of the diethyl ether fraction of cattle urine.

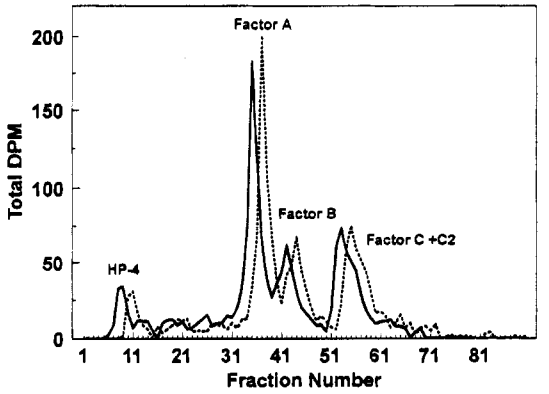

Figure 10. HPLC radiochromatogram comparison of extracts from cattle kidney (—) and cattle liver (- - -). Note: the radiochromatogram for cattle liver is offset by 30 s to allow profile comparisons.

although the other factors were buried within the baseline noise. Overall, the urinary profiles of cattle and rats were similar, with the only difference being more extensive hydrolysis of the ardacin molecule to HP-4 in cattle. Since fortified control stability studies were not performed during the actual collection of the cattle urine, this hydrolysis may still be an artifact of the procedure, which involved larger volumes and more labor-intensive sampling and handling than are required for rats.

The extractable residues from liver and kidney tissues were processed according to the same C_{18} cartridge cleanup procedure as used for urine. The results were consistent, although somewhat variable, across species for the dosed and fortified control tissues, with the 50% ACN/H_2O fractions containing the majority of the eluted radioactivity (26.4–69.4%).

The radiochromatograms for the cattle liver and kidney samples are shown in Figure 10. Both tissues showed nearly identical profiles, with ardacin factors A, B, and C + C_2 clearly visible and comprising 83.6–87.7% of the total sample radioactivity. The relative ratios for the various ardacin factors were very similar

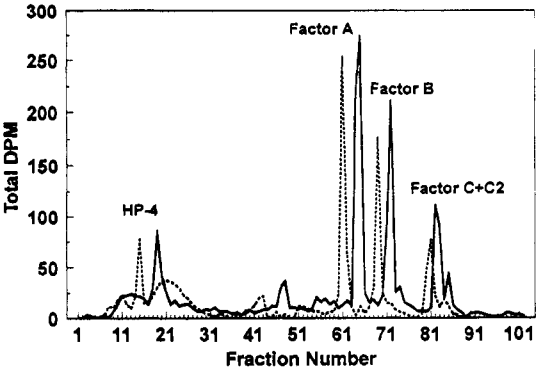

Figure 11. HPLC radiochromatogram of extracts from broiler liver of birds dosed at 15 g/ton, 0-day withdrawal (—), and 50 g/ton, 7-day withdrawal (- - -). Note: the radiochromatogram for 15 g/ton broiler liver is offset by 30 s to allow profile comparisons.

to those found in the fortified control sample, indicating no preferential metabolism or chemical degradation. Following correction for C_{18} recovery and extractability (which were low for tissues), the total radioactivity attributable to ardacin was 19.4% and 7.1% for liver and kidney, respectively. Similar profiles were also seen in the rat liver and kidney samples as well. Unlike cattle urine, very little HP-4 was present in the tissues.

HPLC of the broiler liver tissue extracts showed sharp peaks with excellent resolution among the ardacin factors, due to the slower gradient used for the analyses. Samples from both the 0- (15 g/ton) and 7-day (50 g/ton) withdrawal birds were analyzed, and the resulting radiochromatograms are compared in Figure 11. Once again, identical profiles were obtained in both cases. These results further support the tight binding of ardacin to tissue proteins, which helps to explain its relatively slow depletion from tissues. Even after a 7-day withdrawal period, residues were extractable to approximately the same extent, with parent drug being the only compound present. The total liver residues attributed to intact ardacin for broilers were 20.9% and 26.3% for 0- and 7-day withdrawal birds, respectively.

Assessment of the microbiological activity of the broiler tissue residues is an essential requirement for antibiotic feed additive registration in both Europe and Japan. Extracts from the livers of the birds treated at either 15 or 50 g/ton for 30 days followed by a 7-day withdrawal period were tested according to the agar well method, and the results are shown in Table 3. No detectable activity was found for the 15 g/ton derived extracts as the total extractable residues were below the sensitivity of the method. Measurable activity, however, was obtained for the 50 g/ton derived extracts, which showed microbiologically active residues corresponding to 17.3% of the total liver residues. This value agrees reasonably well with the 26.3% attributable to intact ardacin as determined by HPLC.

Table 3. Microbiological Activity of the Extractable [^{14}C]Ardacin from Broiler Liver (7-Day Withdrawal)

sample	medicated birds (15 g/ton)		medicated birds (50 g/ton)		fortified control[e]
	male	female	male	female	
total residue[a] (ppb)	42.0 ± 9.4	38.5 ± 10.6	197.0 ± 79.6	229.4 ± 89.9	
total extractable residue[b] (ppb)	15.0	9.1	90.2	111.3	95.8
% microbiological activity in extract	ND[c]	ND[c]	32.2	40.1	91.0
microbiological active residue (ppb)	ND[c]	ND[c]	29.0[d]	44.6[d]	87.2[d]

[a] For 15 g/ton birds, (n = 4); for 50 g/ton birds, (n = 6). [b] Total extractable residue = (total residue) × (% extractability). [c] ND, below the limit of detection of 20 ppb. [d] Microbiological active residue = (total extractable residue) × (% microbiological activity). [e] Control liver extract fortified with [^{14}C]ardacin standard at 95.8 ppb. Control extracts without fortification were negative in the assay.

Attempts at HPLC analysis of the broiler kidney extracts were generally not successful. Evidence of the ardacin factors was present; however, total sample radioactivity was too low for the generation of meaningful profiles.

CONCLUSIONS AND HUMAN FOOD SAFETY ASSESSMENT

The results of the ardacin residue/metabolism studies were similar to those reported for other glycopeptide antibiotics including vancomycin (Moellering, 1984) actaplanin (Donoho, 1987), and teicoplanin (Zerilli et al., 1989). This class of compounds is categorized by poor oral absorption and limited metabolism except for hydrolytic losses of sugar and lipid side chains. The comparative metabolism studies, described here, have demonstrated that ardacin is present in all tissues and excreta as the intact molecule across all species profiled. No preferential metabolism or chemical degradation among the ardacin factors was evident, and no sex differences were observed. The small percentage of the ardacin dose that is absorbed becomes rapidly bound (as the intact molecule) to tissue proteins. The equilibration times of 13 and 22 days for cattle and broilers, respectively, are probably the result of the establishment of a steady state between the processes of ardacin protein binding and the depletion of bound residues by normal protein catabolism.

Ardacin toxicology studies indicated a very favorable safety profile (NOEL = 15 mg/kg), and the compound has been assigned Category A by the CVM. Assuming a safety factor of 1000, the maximum permissible residues in muscle for ardacin are 3.0 ppm, with residues of 9.0, 18.0, 18.0, and 0.6 ppm being permitted in liver, kidney, fat, and milk, respectively (CVM, 1994). Acceptance of the recently completed lifetime study will likely reduce this safety factor to 100, resulting in a corresponding 10-fold increase in the above safe concentrations. Since equilibration in cattle and broiler tissues was attained with total residue levels of only 0.032–0.110 ppm and milk residues averaging only 0.002 ppm, a high safety margin is evident across all edible tissues.

On the basis of these results, ardacin has been approved for 0-day withdrawal in both broilers (United States, European Union) and cattle (United States). Since ardacin/broilers was approved for Annex II, the establishment of MRLs was not required for EC registration.

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