[^{14}C]Ceftiofur Sodium Absorption, Distribution, Metabolism, and Excretion in Sheep following Intramuscular Injections

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Six Columbia and mixed-breed sheep (three rams and three females, 33–44 kg) were euthanatized 12 h after the last of 5 intramuscular doses at 2.2 mg of [^{14}C]ceftiofur sodium/kg of body weight. Total dose accountability was 103.5 ± 4.5%: 92.55 ± 4.10% in urine, 6.53 ± 0.85% in feces, 2.40 ± 0.14% in the carcass, and 0.69 ± 0.32% in the intestinal tract. Kidneys were the tissues with highest residue concentration (9.016 ± 1.153 μg/g, 0.29% of the dose). Most residues found in kidney and liver were associated with macromolecules (95.7% and 90.5%, respectively). Of the free residues, all corresponded to polar metabolites devoid of the β-lactam ring necessary for biological activity. Desfuroylceftiofur (DFC)-dimer was the most abundant metabolite (47.58%) found in urine. Polar metabolites devoid of a β-lactam ring comprised 38.5% of the total residues. In plasma, DFC was conjugated to albumin. Free residues containing an intact β-lactam ring were not found. [^{14}C]-Ceftiofur itself was never found in the excreta or plasma or as a tissue residue.

Keywords: *ADME; metabolism; ceftiofur; sheep; NAXCEL*

INTRODUCTION

Third-generation cephalosporin antibiotics have a larger spectrum of activity against Gram-negative bacteria than either first- or second-generation cephalosporins (Neu, 1983; Thornsberry, 1985; Webber and Wheeler, 1982). Ceftiofur sodium (NAXCEL/EXCENEL Sterile Powder) is a third-generation cephalosporin which was approved by the FDA for intramuscular injection for the treatment of certain respiratory diseases in beef cattle, dairy cattle, swine, and day-old chicks (FDA 1988, 1991, 1992). The effectiveness of ceftiofur sodium in the control of Gram-positive and Gram-negative bacterial pathogens of veterinary importance was evaluated both *in vivo* and *in vitro* by Yancey et al. (1987). The metabolism of ceftiofur sodium, which has been described in beef and dairy cattle, rats, and swine (Jaglan et al., 1989, 1992; Gilbertson et al., 1995), differs from that described in humans for other cephalosporins. While cephalosporins which are similar in structure, except for the moieties attached to the 3-position of the dihydrothiazine ring, are excreted primarily in the urine unmetabolized (Machinist et al., 1984; Nakayama et al., 1984; Neu and Shrinivasan, 1981; Patel and Kaplan, 1984), ceftiofur sodium is metabolized to desfuroylceftiofur (DFC), a major metabolite that is biologically active.

The metabolism of [^{14}C]ceftiofur sodium in sheep is described in this paper.

EXPERIMENTAL PROCEDURES

Test Materials. Ceftiofur sodium, radiolabeled with ^{14}C in the 2-position of the thiazole ring, was synthesized by G. A. Hoffman of the Upjohn Co. and was provided as dry powder formulated with NAXCEL in 10 separate vials, each containing about 400 mg of [^{14}C]ceftiofur sodium (specific activity ~ 1.3 μCi/mg). Radiochemical purity was determined by high-performance liquid chromatography (HPLC) to be >98% using the HPLC conditions described under Dose Preparation.

Other Compounds. Other compounds used were synthesized by C. L. Gatchell of the Upjohn Co. following previously described techniques (Gilbertson et al., 1995) and are described in Table 1 along with the approximate retention time in the different HPLC solvent systems used (see HPLC Conditions).

Animal Phase. Three Columbia breed rams about 5 months old, weighing 38–44 kg, and three mixed-breed (Columbia, Hampshire, Suffolk) female sheep about 4–5 months old, weighing 33–41 kg, were acclimated to the laboratory and handling for 3 weeks. The sheep were fed 700 g of concentrate (Northern Crops Institute, North Dakota State University) daily and mixed hay and water *ad libitum*. The day before treatment the sheep were put in metabolism cages in a room artificially lighted (7 a.m. to 9 p.m.), of ambient humidity and temperature (about 22 °C). The drug was injected intramuscularly (IM) in the posterior of left and right rear legs. The first through fourth doses were injected in the left leg, and the fifth dose was injected in the right leg. The average dose for male sheep was 11.53 ± 0.09 mg of [^{14}C]-ceftiofur/kg of body weight (bw), which calculated to be 2.3 mg of [^{14}C]ceftiofur/kg of bw per day, whereas the average dose for female sheep was 11.02 ± 0.13 mg of [^{14}C]ceftiofur /kg bw, which calculated to be 2.2 mg of [^{14}C]ceftiofur/kg of bw per day. The average daily dose for all sheep was 2.25 mg of [^{14}C]-ceftiofur/kg for 5 consecutive days.

Dose Preparation. On the day of use, the contents of one vial were dissolved in 8 mL of sterile water. The solution was assayed in triplicate for ^{14}C and an aliquot from each vial was also monitored for purity by HPLC. The HPLC system was equipped with both a radioactivity monitor and a UV detector (254 nm). A DuPont Zorbax C_8 (4.6 × 250 mm) was used with an isocratic mobile phase that contained 7.7 g of ammonium acetate and 27 mL of tetrabutylammonium hydroxide in 1400 mL of water (adjusted to pH 6.6–6.8 with acetic acid), 600 mL of methanol, and 320 mL of tetrahydrofuran. Results of the radioactive assays demonstrated that the daily doses were homogeneous solutions containing the intended amount of ^{14}C

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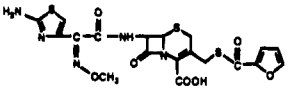
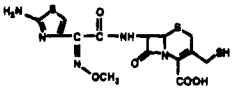
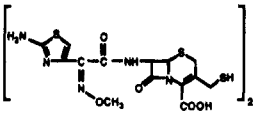
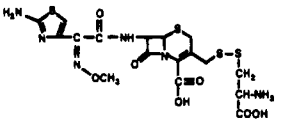
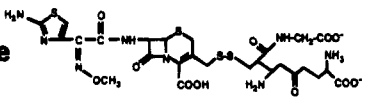
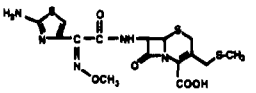
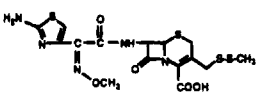
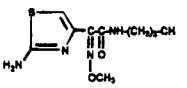
Table 1. Name, Structure, and Retention Times in the Different HPLC Systems of the Compounds Described in the Text

Common Name	Abbreviation	Structure	Retention Time (min) HPLC System[a]			
			1	2	3	4
Ceftiofur (Syn Δ^3)	CF		52.4	62.8	ND	50.3
Desfuroylceftiofur	DFC		31.5	48.5	ND	31.5
3,3'-Desfuroylcefitiofur Disulfide	DFC-Dimer		50.8	57.4	38.1	49.3
Desfuroylcefitiofur Cysteine Disulfide	DFC-Cysteine		16.2	41.5	27.1	24.5
Desfuroylcefitiofur Glutathione Disulfide	DFC-Glutathione		18.0	42.8	29.3	29.3
Desfuroylcefitiofur -S-Methyl	DFC-S-Methyl		ND	ND	26.3	ND
Desfuroylcefitiofur -S-S-Methyl	DFC-S-S-Methyl		ND	ND	32.2	ND
2-(2-Aminothiazole-4-yL)-2-Methoxyiminoacetic Acid	Polar C		12	ND	ND	ND

with the exception of the last dose for all females, which contained approximately 50% of the intended amount of ^{14}C. This did not affect [^{14}C]ceftiofur recovery, because recovery was based on ^{14}C balance, or plasma and urine recoveries and kinetics, because the specific activity from each day was used in the calculations. Effects on the concentrations of ^{14}C found in tissues are believed to be nil since ceftiofur was rapidly metabolized and eliminated; thus, residues found in tissues were probably the result of accumulation from all doses. The data support the above statement since tissue residue concentration differences were not observed between sexes. A sex effect on tissue residue concentrations has never been observed (Jaglan et al., 1989, 1992; Gilbertson, 1995).

Sample Collection. Urine and feces were separately collected and weighed at 24-h intervals, except for the fifth day, on which the time period varied from greater than 10 h to less than 12 h. Urine was collected through catheters in the female sheep and through urinals on the male sheep. Blood was collected from the jugular vein at 0, 1, 2, 4, 8, 12, and 24 h after each dose, except after the last dose (no 24 h collection). Plasma was harvested from an aliquot of blood and frozen until assayed. The cerebrospinal fluid was collected

after the sheep were anesthetized with xylazine (Rompun; 0.22 mg/kg of bw; Haver-Mobay, Shawnee, KS) and ketamine (Ketaset; 22.0 mg/kg of bw; Aveco, Fort Dodge, IA). The anesthetized sheep were killed by exsanguination. The following tissues were removed for analysis: longissimus muscle, injection sites, front feet, head, testicles, adrenals, kidneys, fat, heart, lungs, liver, spleen, intestines, mesenteric lymph nodes, omasum/abomasum, rumen/reticulum, brain, and tonsils. The remainder of the animal, "carcass", was then ground and sampled.

Instrumental Analysis. *Electrospray Mass Spectrum.* A Vestec electrospray mass spectrometer was used with the following running conditions: the needle was set at 2.01 kV, the ion current at 0.130 μA, the nozzle at 175 V, the repeller at 15 V, and the block at 235 °C. Mobile phase 1 (acidic conditions) was 5% acetic acid in 1:1 methanol/water with a flow rate of 3 μL/min and a 5 μL loop injection. Mobile phase 2 (neutral conditions) was 0.01 M ammonium acetate (pH 7.4) with a flow rate of 3 μL/min and a 5 μL loop injection. The albumin standard used was sheep albumin fraction V (powder), Sigma product no. A3264.

Thermospray Mass Spectrum. The thermospray interface was a Nermag/Vestec system/controller (Vestec Corp., Houston, TX), operated at various source and vaporizer temperature settings and repeller voltages. The mass spectrometer was a Nermag resolver R 10-10 L quadrupole instrument, 2000 amu, fitted with the Nermag/Vestec thermospray source and probe, set to operate in the ions$^+$ mode. The MS controlling software/hardware was the PC (OS/2) based Teknivent Vector/Two GC/LC/MS Workstation, V1.4 (Teknivent, Maryland Heights, MO).

During the analysis of samples, the acquisition was set for selected ion monitoring (SIM) at various combinations of the following ions: m/z 201, 241, 243, 259, 285, 316, 325, 341, 385, 386, 412, and 430 with a 0.5 amu wide window centered on each ion and a dwell time of 10 or 15 counts for each ion.

HPLC Systems. Two HPLC systems were used. The first one consisted of a Varian Model 5500 VISTA HPLC pump equipped with a Varian UV detector (254 nm), a Radiomatic FLO-ONE/Beta Model A-280 radioactive flow detector, and a Bio-Rad AS-100 HRLC automatic sampling system (5 °C). The second one consisted of a Waters 600 E powerline multisolvent delivery system, controller and pump equipped with a 717 Plus autosampler and a 486 tunable absorbance detector (254) nm, and a Radiomatic FLO-ONE/Beta Model A-500 radioactive flow detector and processing software.

HPLC Conditions (See Table 1). System 1. A Burdick and Jackson (250 × 4.6 mm), HLD OC5 octyl, 5 μm column was used. The mobile phases were the following: A, 0.01 M ammonium acetate (pH 5.0); B, 6:4 methanol/water. The gradient was linear 0–20% B from 0 to 10 min, remained isocratic (20% B) from 10 to 35 min, increased linearly to 100% B from 35 to 45 min, and remained isocratic (100% B) from 45 to 55 min. The flow was 1 mL/min. The column was equilibrated with the starting conditions for 10 min before the next injection.

System 2. A Brownlee Lab (220 × 4.6 mm) RP-18 5 μm column was used. The mobile phases were the following: A, 0.01 M ammonium acetate (pH 5.0); B, 6:4 methanol/water. Conditions were isocratic with 0% B from 0 to 15 min, the gradient was linear 0–39% B from 15 to 40 min, increased linearly to 100% B from 40 to 65 min, and remained isocratic at 100% B from 65 to 75 min. The flow was 1 mL/min. The column was equilibrated with the starting conditions for 10 min before the next injection.

System 3. A Keystone Scientific (250 × 4.6 mm) BDS Hypersil C_{18} 5 μm column was used. The mobile phases were the following: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile. The gradient was linear 0–35% B from 0 to 35 min and 35–50% B from 35 to 50 min; conditions remained isocratic (50% B) from 50 to 55 min. The flow was 1 mL/min. The column was equilibrated with the starting conditions for 25 min before the next injection.

System 4. A Bakerbond Wide-Pore (100 × 4.6 mm) octyl (C_8) 5 μm column was used. The mobile phases were the following: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile. Conditons were isocratic (0% B) from 0 to 15 min; the gradient increased linearly 0–12% B from 15 to 25 min, 12–16% B from 25 to 45 min, and 16–90% B from 45 to 55 min; conditions remained isocratic (90% B) from 55 to 65 min. The flow was 1 mL/min. The column was equilibrated with the starting conditions for 10 min before the next injection.

Total Radioactivity Determination. Total radioactivity was determined by combustion analysis (Packard Model 307 oxidizer) of triplicate weighed aliquots of the following specimens: urine (about 0.1 g), blood samples (about 0.4 g), homogenized feces (2 parts feces and 1 part water, about 0.5 g combusted), bile, gastrointestinal contents (about 0.5 g), and all other tissues except for fat (between 0.1 and 0.5 g), followed by ^{14}C counting in a Packard Model 1600 liquid scintillation spectrometer (Packard Instrument Co., Meriden, CT). Plasma (0.2 mL) and cerebrospinal fluid (0.2 mL) were counted directly after 10 mL of Ecolite+ counting cocktail (ICN, Costa Mesa, CA) was added. Adipose tissue (visceral) was weighed (about 0.1 g) directly into counting vials to which 9 mL of Carbosorb was added. After the vials had stood overnight at room temperature, 12 mL of Permafluor was added, the vials were shaken vigorously, and the ^{14}C was counted in the Packard 1600 liquid scintillation counter.

Preparation of Samples for Metabolite Profile. *Tissues.* Kidney (10 g) and liver (20 g) from each sheep were homogenized with 20 mL of 1% acetic acid and centrifuged at 48 000 RCF for 40 min. A 500 μL aliquot of each supernatant was analyzed (in duplicate) for total radioactivity. The remaining supernatant was transferred to individual Amicon Centriprep 10 units (Model 4304) and centrifuged at 3000 RCF for 2 h at 4 °C. Each filtrate, which contained radioactivity not associated with macromolecules, was lyophilized. The dry residue was redissolved in 2 mL of 0.01 M ammonium acetate (pH 5), and duplicate 100 μL aliquots were analyzed for radioactivity. The remaining solution was analyzed by HPLC using system 1. Insufficient activity was found in the muscle to allow radioactive determination following HPLC with the sensitivity of the instruments available.

Urine. Individual urine samples were diluted (1:1) with 0.01 M ammonium acetate (pH 5.0), filtered (Acrodisc filter, 0.45 μm), and analyzed by HPLC (system 2). To achieve better separation between peaks for fraction collection, a pooled source of urine was filtered (Acrodisc filter, 0.45 μm) and analyzed by HPLC (system 3). Radiolabeled peaks were purified by collecting fractions from repeated injections. Radioactive fractions with similar retention times were pooled and lyophilized. The identities of the fractions were confirmed by thermospray MS. Desfuroylceftiofur (DFC)-containing metabolites were identified by monitoring the disappearance of the radiolabeled peaks associated with these metabolites and the corresponding appearance of a radiolabeled peak corresponding to free DFC, which resulted from the cleaving action of excess dithioerythritol (DTE) in 0.1 M ammonium acetate (pH 8.7) at 50 °C for 2 h.

Plasma. Plasma samples were diluted (1:1) with 0.1% trifluoroacetic acid (TFA) in water, filtered (0.45 μm), and analyzed by HPLC (system 4). Using a 1:1 dilution, plasma samples were still too dense for optimum HPLC analysis; further dilution, however, was not possible due to the low radioactivity present in plasma. The radiolabeled peak that eluted at ~54–55 min was collected from several HPLC injections of a pooled plasma source, concentrated under a stream of nitrogen, and analyzed by electrospray MS. To monitor for free residues, radioactivity bound to proteins was precipitated with acetonitrile (1:1). The acetonitrile layer, which was devoid of protein, was dried under nitrogen, redissolved in 1% TFA in water, and analyzed by HPLC (system 4). To determine which radiolabeled metabolite was conjugated to the protein fraction, a pooled source of plasma was incubated with DTE for 15 min at 50 °C and analyzed by HPLC (system 4). The only radiolabeled peak observed after HPLC analysis of plasma samples had a large variability in retention time between samples (~1 min). To confirm that it did not correspond to free ceftiofur, cochromatographic analysis with a pooled source of incurred plasma spiked with approximately the same activity (~600 dpm) of [^{14}C]ceftiofur was conducted using HPLC system 4.

Table 2. Area under the Concentration Curve (AUC_{0-12}), Maximum Observed Concentration (C_{max}), and Concentration 12 h after Dosing (C_{12h}) of Total [^{14}C]Ceftiofur Residues in Plasma after Five Intramuscular Injections at 24 h Intervals at a 2.2 mg/kg of Body Weight Dose Level[a]

	AUC_{0-12} (μg h/mL)			C_{12h} (μg/mL)			C_{max} (μg/mL)	
day	av	SD	P value[b]	av	SD	P value[b]	av	SD
1	34.88	5.68		0.87	0.23		8.51	2.05
2	37.53	4.01	0.21	1.12	0.23	0.08	8.32	0.69
3	38.76	4.24	0.07	1.28	0.18	0.01	8.11	0.60
4	41.87	1.47	0.003	1.38	0.16	0.002	8.61	0.86
5	47.80	5.73	0.0001	2.05	0.53	0.0001	9.09	1.01

[a] Average over six sheep. [b] P value compared to day 1.

Statistical Analyses. *Plasma.* The area under the drug concentration curve from time 0 to 12 h (AUC_{0-12}) was calculated using the trapezoidal rule. For each animal at each time of serum sampling, three replicate samples were obtained. For the purposes of analysis, a mean of these observations was calculated and used in all subsequent calculations. The statistical model fit to the AUC_{0-12} values was a two-factor analysis of variance in which animal was one factor and day of dosing was the second factor. Comparison between the average day 1 value and subsequent days was performed using a t test and the error term from the analysis of variance. A single degree of freedom statistical test for linear trend was performed using the method of Winer (1971). The 12 h serum concentrations and the peak concentration value for each animal and each day were obtained directly from the observed values and were statistically analyzed using the model described for the AUC data.

Urine. The data were analyzed using a two-factor analysis of variance in which animal served as the blocking factor and day of sample was the other factor. The sampling days were compared to one another using a t test with the error term derived from the model specified above.

RESULTS AND DISCUSSION

Excretion of Radioactive Dose and Residues in Tissues. Excretion studies following five IM injections at 24 h intervals in sheep at 2.2 mg of [^{14}C]ceftiofur/kg of bw indicated that 92.55 ± 5.20% of the dose was recovered in urine and 6.53 ± 0.85% was recovered in feces (Table 2). Recoveries in sheep urine were higher than the previously reported recoveries of 62.8 ± 7.6 and 61.82 ± 4.70% in urine of dairy cattle and swine, respectively (Jaglan et al., 1992; Gilbertson et al., 1995), indicating that sheep clear more of the drug through the kidneys than cattle and swine. Recoveries in sheep feces, however, were lower than the 35.7 ± 9.6 and 10.75 ± 5.07% previously reported in dairy cattle and swine, respectively (Jaglan et al., 1992; Gilbertson et al., 1995).

The carcass accounted for 2.40 ± 0.14% of the dose and the intestinal tract for 0.69 ± 0.32%. Total accountability of the dose was 103.5 ± 5.00%, including the cage rinse. No radioactivity was detected in brain tissue or cerebrospinal fluid.

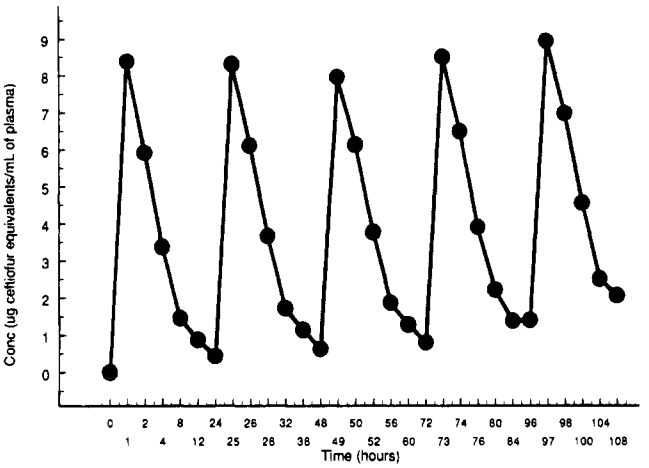


Figure 1. Average ^{14}C residue levels expressed as ceftiofur sodium equivalents (micrograms) per milliliter of plasma vs time (average over six sheep).

At 12 h after the last dose, the highest concentration was found in the kidney with an average concentration of 9.02 ± 1.15 μg/g (Table 3). The second highest concentration was found in the fifth injection site (1.07 ± 0.43 μg/g), followed by the lungs (0.63 ± 0.12 μg/g), then the fourth injection site (0.55 ± 0.08 μg/g) and the heart (0.34 ± 0.07 μg/g). Next in concentration was the muscle, the organ meat most consumed by the public, with an average of 0.13 ± 0.02 μg/g, and the fat (0.12 ± 0.03 μg/g).

Residues in Plasma and Blood. The average ^{14}C residue levels expressed as ceftiofur sodium equivalents (micrograms) per milliliter of plasma vs time are displayed in Figure 1. A statistical analysis of the AUC_{0-12} indicated that the AUC_{0-12} tended to increase with each subsequent day of dosing. Statistical differences appeared between the day 1 and the day 4 average AUC_{0-12} ($P = 0.003$) and between day 1 and day 5 ($P = 0.0001$) (Table 4). The plasma residue concentration at 12 h postdosing also revealed statistically significant effects due to day of dosing, with C_{12h} of days 3–5 being significantly higher than that of day 1 ($P < 0.01$) (Table 4). The maximum average plasma concentrations, which were observed at 1 h after each dose, showed no statistically significant differences. Note that only one sample was obtained around the maximum concetration (1 h); thus, the exact time at which the maximum concentration of residues in plasma occurred is unknown and cannot be predicted. This could explain why no statistically significant differences were observed between the maximum average plasma concentrations.

The total ^{14}C residue levels in blood followed a profile similar to that of plasma but were on the average 27–35% lower, indicating that very little radioactivity was present in the erythrocytes, since the packed cell volumes of the blood samples were 25–38%.

Table 3. Recovery of ^{14}C from Sheep 12 h after the Last of Five Intramuscular Injections at 24 h Intervals (Percent of Dose)

sheep	dosage	% of dose recovered								
no.	(mg)	urine	feces	carcass	int. tract	kidneys	liver	other tissues	cage rinse	total
3062	441.16	89.8	5.2	2.33	1.34	0.29	0.14	0.49	0.26	99.8
3129	509.54	89.9	6.5	2.23	0.62	0.23	0.09	0.52	0.66	100.5
3245	450.91	91.4	6.0	2.64	0.60	0.29	0.16	0.65	0.19	101.9
3409	422.01	92.3	7.6	2.41	0.58	0.30	0.11	0.56	0.36	104.4
3468	462.27	100.7	7.1	2.44	0.52	0.30	0.10	0.44	0.41	112.1
3489	402.27	91.2	6.8	2.35	0.50	0.34	0.10	0.42	0.19	102.3
av ± SD		92.55 ± 5.2	6.53 ± 0.85	2.40 ± 0.14	0.69 ± 0.32	0.29 ± 0.04	0.12 ± 0.03	0.51 ± 0.08	0.35 ± 0.18	103.5 ± 5.2

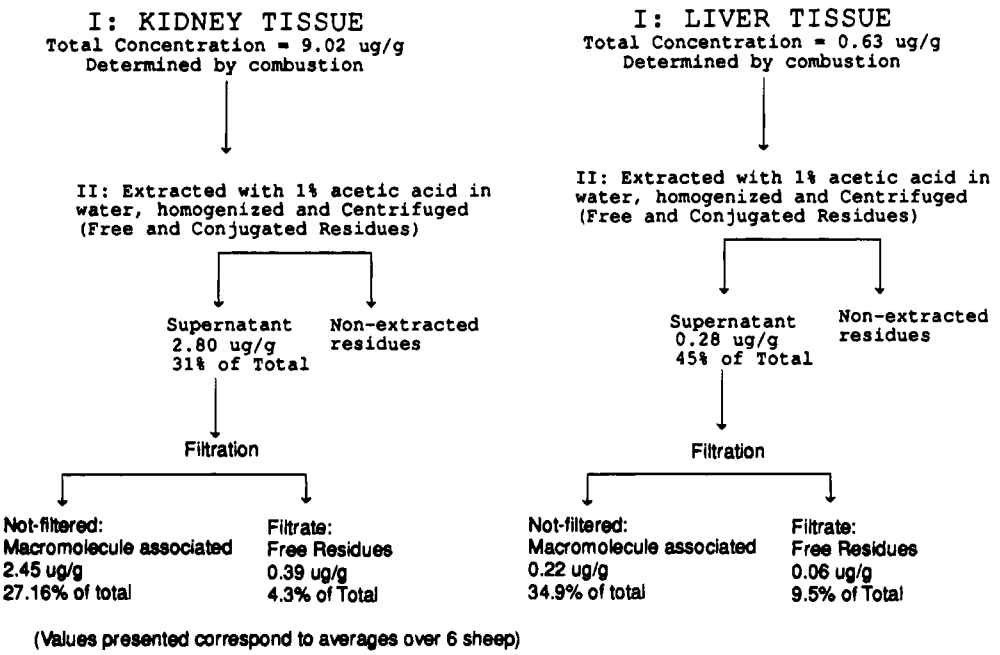


Figure 2. Recoveries of ceftiofur and related residues in sheep kidney and liver.

Table 4. Total Residue Levels of [14C]Ceftiofur Free Acid Equivalents (Micrograms per Gram) in Edible Tissues of Sheep at 12 h after the Last of Five Intramuscular Injections at 2.2 mg/kg

sheep no.	sex	kidney	muscle	liver	fat	5th inj site	4th inj site	lungs	heart
3062	M	9.64	0.15	0.80	0.13	0.60	0.58	0.73	0.40
3129	M	6.91	0.11	0.42	0.12	1.53	0.44	0.52	0.31
3245	M	9.63	0.16	0.79	0.18	1.28	0.54	0.81	0.43
3409	F	8.77	0.11	0.60	0.09	1.40	0.66	0.53	0.31
3468	F	8.94	0.12	0.50	0.10	1.09	0.51	0.55	0.25
3489	F	10.20	0.12	0.62	0.12	0.51	0.55	0.61	0.35
av ± SD		9.02 ± 1.15	0.13 ± 0.02	0.62 ± 0.15	0.12 ± 0.03	1.07 ± 0.43	0.55 ± 0.08	0.63 ± 0.12	0.34 ± 0.07

The Nature of Kidney Metabolites. An average of 0.29 ± 0.04% of the original ^{14}C dose was present in the kidneys at a concentration of 2.80 (± 0.39) μg of ceftiofur equivalents/g of tissue. Upon homogenization and centrifugation, 31.0 ± 2.93% of this residue (2.80 ± 0.39 μg of ceftiofur equivalents/g of tissue) was recovered in the supernatant, while the remaining was associated with macromolecules and was not extracted from the kidney matrix by the procedure used (Figure 2). The extracted fraction consisted of free ceftiofur-related residues (0.39 ± 0.1 μg of ceftiofur equivalents/g of tissue) and ceftiofur-related residues associated with macromolecules (2.45 ± 0.33 μg of ceftiofur equivalents/g of tissue).

Upon HPLC analysis (system 1) of the free ceftiofur-related residues fraction in the kidney, two radioactive peaks were detected in the six samples analyzed (Figure 3). The elution time of these peaks did not correspond to the elution time of any of the ceftiofur-related metabolite standards which contained an intact β-lactam ring (Table 1). The second radioactive peak eluted at the same retention time as the previously characterized polar C.

The Nature of Liver Metabolites. An average of 0.12% of the original [14C]ceftiofur dose was present in the liver at a concentration of 0.63 μg of ceftiofur equivalents/g of tissue. Upon homogenization and centrifugation, 45.0 ± 7.7% of this residue (0.28 ± 0.07 μg of ceftiofur equivalents/g of tissue) was recovered in the supernatant, while the remaining was associated with macromolecules and was not extracted from the liver matrix by the procedure used (Figure 2). The extracted fraction consisted of free ceftiofur-related residues (0.06 ± 0.01 μg of ceftiofur equivalents/g of tissue) and ceftiofur-related residues associated with macromolecules (0.22 ± 0.06 μg of ceftiofur equivalents/g of tissue).

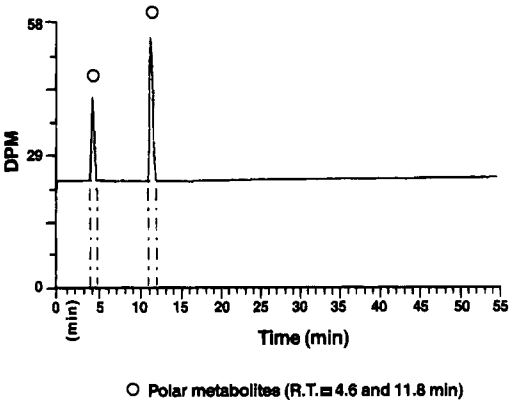


Figure 3. Representative HPLC ^{14}C chromatogram of sheep kidney extract not associated with macromolecules. Chromatogram was obtained using HPLC system 1.

Upon HPLC analysis (system 1) of the free ceftiofur-related residues fraction in the liver, one major radioactive peak (retention time 2.10 min) was detected in the six samples analyzed. The elution volume of this product did not correspond to the retention time of any of the ceftiofur-related metabolite standards which contained an intact β-lactam ring or to the polar C metabolite. Due to its early elution volume, it was considered likely that this was a small polar molecule.

The Nature of Urinary Metabolites. Seven radioactive peaks were detected upon HPLC analysis

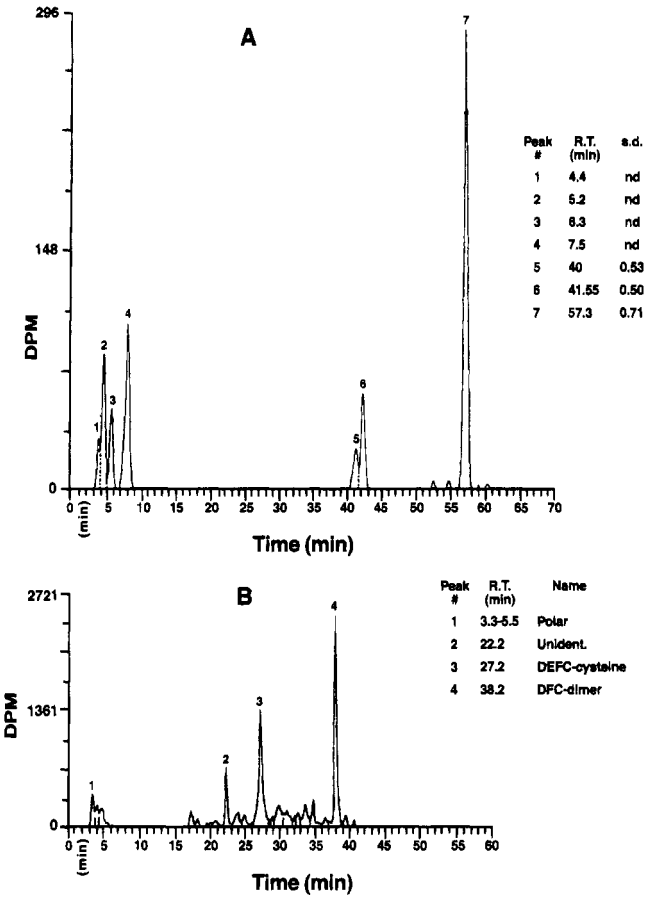


Figure 4. Representative HPLC ^{14}C chromatograms of sheep urine obtained using HPLC system 2 (A) and HPLC system 3 (B).

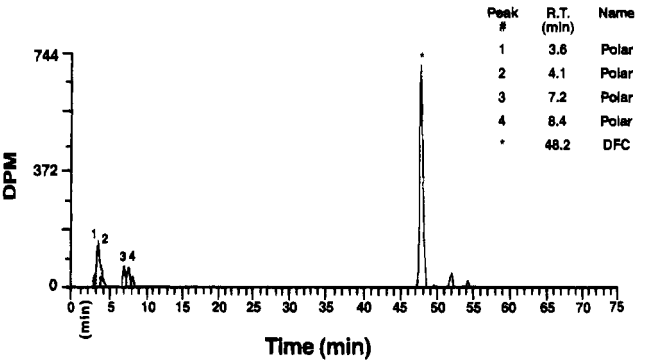


Figure 5. Representative HPLC ^{14}C chromatograms of sheep urine after treatment with the reducing agent dithioerythritol (DTE). Chromatogram was obtained using HPLC system 2.

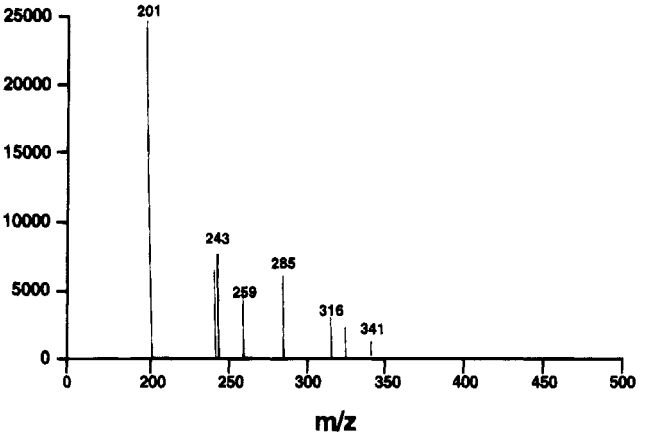


Figure 6. SIM-thermospray MS of the unidentified urine metabolite eluting as peak 5.

(method 2) of the urine samples. The four radioactive peaks labeled 1−4 were attributed to polar metabolites of ceftiofur due to their early elution time (less than 15 min) from the C_{18} HPLC column. The radioactive peaks labeled 5, 6, and 7 had average retention times of 40.0 ± 0.6, 41.6 ± 0.5, and 56.8 ± 0.7 min, respectively. According to the retention times of the ceftiofur-related metabolite standards (Table 1), peaks 6 and 7 were tentatively assigned to the DFC-cysteine and DFC-dimer, respectively. When using HPLC system 2, the average retention time of peak 5 could not be matched with the retention time of any of the known ceftiofur-related metabolites. Also, because peaks 5 and 6 eluted with a small difference in retention time (1.5 min) and had a large variability in retention times between samples (~0.5 min), pooled sample sources were analyzed using HPLC system 3. Using the new chromatographic conditions, better separation between peaks 5 and 6 (Figure 4B) and more consistency in retention times were achieved. The new average retention times were 22.0 and 27.2 min for peaks 5 and 6, respectively. With HPLC system 2, the retention time of peak 5 was not similar to that of any of the known ceftiofur metabolites, nor to that of DFC-S-methyl or DFC-S-S-methyl, which eluted at 26.3 and 32.2 min, respectively. The retention time of peak 6 corresponded to that of the DFC-cysteine conjugate. The retention time of peak 7 was 38.2 min and corresponded to that of the DFC-dimer standard.

The radioactive signals due to peaks 5−7 disappeared upon treatment of the samples with reducing agent dithioerythritol (DTE), indicating the disulfide bond of the conjugates and dimer was cleaved to desfuroylceftiofur. A representative chromatogram of a urine sample treated with DTE (Figure 5) shows the disappearance of peaks 5−7 and the appearance of a new peak with the retention time of desfuroylceftiofur. The retention times of radioactive peaks 1−4 were unaffected by treatment with DTE, indicating that these residues were devoid of the desfuroylceftiofur moiety necessary for biological activity.

The identities of peaks 5−7 were confirmed by thermospray MS. The major ions present in the thermospray MS of the DFC-dimer (m/z 201, 243, 325, and 385) were found in peak 7 with approximately the same relative intensities and elution time. The major ions present in the thermospray MS of the DFC-cysteine standard (m/z 201, 243, 259, 285, 325, and 386) were found in peak 6 with approximately the same relative intensities and elution time. The thermospray spectrum of the unidentified metabolite, peak 5, contained ions indicative of the presence of a DFC moiety (m/z 201, 243, 259, 285, 316, 325, and 341, Figure 6).

Table 5 lists the percentages of ^{14}C polar metabolites and of metabolites containing an intact β-lactam ring that were found in the urine of the six sheep at 24, 48, 72, 96, and 108 h after the first dose. There was large variability between animals. The average percentage of polar metabolites per animal ranged between 27 and 54%, with an average of 38.5%. For the metabolites containing an intact β-lactam ring, the average percentage per animal ranged between 46 and 73%, with an average of 61%. The average percentage of polar metabolites per day appeared to increase with time. This difference, however, was not statistically significant ($P > 0.99$), and there was large variability between animals ($P < 0.01$). The reverse was true for the DFC-

Table 5. Percent[a] of Various Metabolites Found in the Urine of Sheep following Multiple Intramuscular Injections of Ceftiofur Sodium

	animal (sex)													
	3062 (M)		3129 (M)		3245 (M)		3409 (F)		3468 (F)		3489 (F)		av[c]	
hours[b]	% polar	% conjugated	% polar	% conjugated	% polar	% conjugated	% polar	% conjugated	% polar	% conjugated	% polar	% conjugated	% polar	% conjugated
24	61	39	34	66	39	61	28	72	29	71	39	66	37	63
48	54	46	35	65	29	71	27	73	53	47	31	69	38	62
72	51	49	38	62	44	56	32	68	38	62	25	75	38	62
96	49	51	47	53	43	57	33	67	44	56	22	78	39	60
108[e]	56	44	43	57	56	44	39	61	21	79	20	80	39	60
av[d]	54	46	39	61	42	58	32	68	37	63	27	73		
SD	4.7		5.4		9.7		5.0		12.5		7.7			

[a] Percent of total eluted from HPLC. [b] Hours after first treatment. [c] Means by day. [d] Means by animal. [e] Collections 12 h after last treatment.

containing metabolites: the average percentage of DFC-containing metabolites appeared to decrease over time, though the difference was not statistically significant ($P > 0.99$).

Table 6 lists the metabolites containing an intact β-lactam ring as a percentage of the total ^{14}C residues found in the urine of the six sheep at 24, 48, 72, 96, and 108 h after the first dose. The average percentage of unidentified metabolite excreted per animal ranged between 0 and 6.8%, with an average of 5.1%. For DFC-cysteine and DFC-dimer, the average percentage excreted per animal ranged between 0 and 13.5% and between 34 and 68.2%, with averages of 8.73 and 47.58%, respectively.

The daily average percentage of the unidentified metabolite and DFC-cysteine appeared to increase from 24 to 96 h. DFC-dimer appeared to decrease in the same period of time. These differences were not statistically significant ($P > 0.4$) for all cases, and there was large variability between animals ($P < 0.01$).

Plasma Metabolite Profile. *Nature of Plasma Metabolites.* The HPLC radioactive profile of the six plasma samples obtained 1 h after the first injection and the six obtained at slaughter time (12 h after the fifth dose) indicated the presence of only one radioactive peak. The retention time of this peak was variable and increased gradually from 52 to 56 min with successive injections (Figure 7A). To eliminate the possibility that it could be due to ceftiofur, the standard molecule that had the closest retention time, a cochromatography analysis was performed. Both peaks were clearly resolved after chromatography of radiolabeled plasma fortified with [^{14}C]ceftiofur, indicating that the radioactive peak present in plasma corresponded to a radiolabeled fragment probably bound to protein. The difference in retention time between both peaks was 1.5 min. All other known ceftiofur-related residues had a retention time of less than 50 min under these chromatographic conditions (Table 1).

No radioactivity was observed in the chromatograms of supernatants obtained after precipitation of the protein fraction of plasma samples with acetonitrile. Thus, all of the detectable radioactivity was precipitated with the protein fraction and, therefore, was likely to be associated with it. DFC was released upon incubation of a pooled source of plasma with DTE. No other free [^{14}C]ceftiofur-related residues were found after DTE incubation.

Plasma samples collected 24 h after the first dose were pooled, and plasma samples collected 1 h after the second dose were pooled and injected undiluted into the HPLC. Very little activity was found in the first sample (24 h), indicating that most of the ^{14}C label had been absorbed from the blood. Most of the ^{14}C label present in plasma at this time was due to early eluting peaks, hence, polar metabolites (89%). The rest of the recovered radioactivity (11%) corresponded to a bound fraction (Figure 7). HPLC analysis of the sample (1 h after the second dose) indicated the presence of one major peak that corresponded to a protein-bound fraction. HPLC analysis of undiluted samples resulted in high background radioactivity; thus, it is possible that a small percentage of polar metabolites was present in the second sample (1 h after the second dose).

Characterization of the Fragment Associated with Radioactivity. The electrospray mass spectrum of the radioactive peak found in plasma and that eluted between 52 and 56 min in HPLC system 4 indicated the presence of one major component with an average molecular mass of 66412.1 g, which corresponded to sheep serum albumin. Spectrum characteristics and the calculated molecular mass were in agreement with the electrospray mass spectrum of a commercial sheep serum albumin (66411.5 g). It was expected that under the acid conditions used for thermospray analysis the [^{14}C]ceftiofur-related residues would be cleaved from the protein.

CONCLUSIONS

Sheep differ from the other species that have been studied (Jaglan et al., 1989; Gilbertson, 1995) in that they eliminate more ceftiofur metabolites via the urine. In this study, the injected ^{14}C moved rapidly into the blood of the sheep and was rapidly eliminated, mostly in the urine (~92.55%). The tissue where highest residue concentrations were found 12 h after the last dose was the kidney (9.02 μg/g, 0.29% of the total dose). The residue concentrations in the kidneys averaged 8.4 times greater than those found in the sites of the last injected dose, which were the tissues with the next highest residue concentration. Recovery of the ^{14}C in brain and cerebrospinal fluid was nil, indicating very little movement of ceftiofur across the blood–brain barrier. Total recovery of ^{14}C for the study was approximately 100%. The total ceftiofur-related residues in tissues 12 h after the last of five intramuscular injections at 24 h intervals at 2.2 mg/kg were lower than half the concentration defined by the Food and Drug Administration (1991) as safe.

Table 6. Metabolites Containing an Intact β-Lactam Ring, Expressed as a Percentage of the Total ^{14}C Residues Found in the Urine of Six Sheep at 24, 48, 72, 96, and 108 h after the First of Five Intramuscular Injections of Ceftiofur Sodium

	animal (sex)																			
	3062 (M)			3129 (M)			3245 (M)			3409 (F)			3468 (F)			3489 (F)			av[b]		
hours[a]	% un-ID[c]	% DFC-cyst	% DFC-dimer	% un-ID[c]	% DFC-cyst	% DFC-dimer	% un-ID[c]	% DFC-cyst	% DFC-dimer	% un-ID[c]	% DFC-cyst	% DFC-dimer	% un-ID[c]	% DFC-cyst	% DFC-dimer	% un-ID[c]	% DFC-cyst	% DFC-dimer	% un-ID[c]	% DFC-cyst	% DFC-dimer
24	10	0	29	5	13	48	4	12	46	0	0	72	9	15	48	4	11	51	5.5	8.4	49.0
48	10	0	36	7	15	43	10	12	49	0	0	73	3	10	34	2	12	55	5.4	8.1	48.4
72	4	8	36	9	11	42	8	11	37	0	0	68	9	13	41	6	10	59	5.8	8.9	47.2
96	6	11	35	5	8	40	7	12	37	0	0	67	7	15	34	11	56	78	6.0	9.6	44.9
108[d]	2	7	34	0	10	47	4	10	30	0	0	61	7	14	58	6	10	64	3.1	8.7	48.9
av[e]	6.5	5.4	34.0	5.2	11.4	44.0	6.6	11.2	39.7			68.2	6.8	13.5	42.9	5.8	10.9	56.7	5.15	8.73	47.58
SD	3.5	5.1	2.8	3.3	2.5	3.4	2.7	0.9	7.4			5.0	2.5	2.0	10.2	3.3	1.2	5.0	2.59	5.06	12.57

[a] Hours after first treatment. [b] Means by day. [c] Means by animal. [d] Collections 12 h after last treatment. [e] Unidentified.

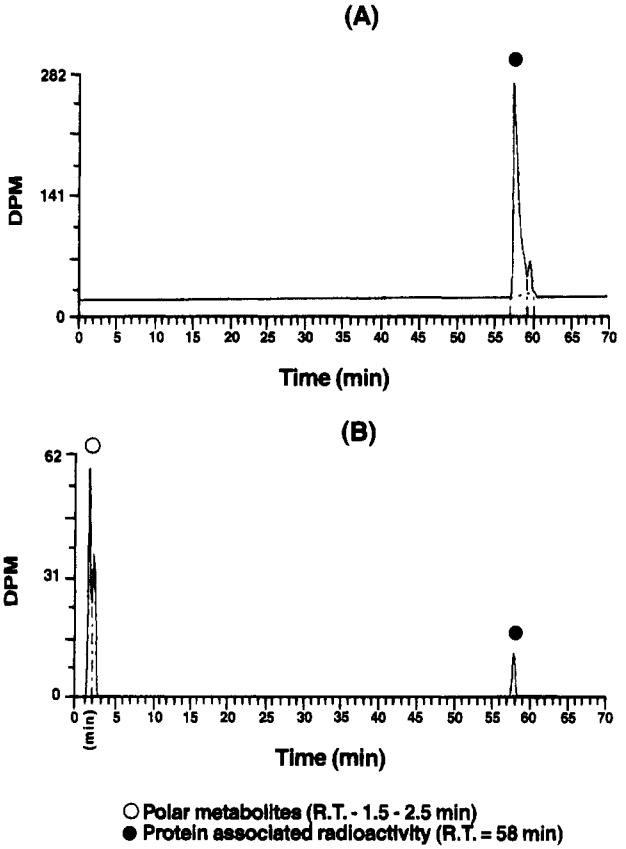


Figure 7. HPLC ^{14}C chromatogram of sheep plasma obtained 1 (A) and 24 h (B) after first dose. Chromatogram was obtained using HPLC system 4.

Parent [^{14}C]ceftiofur itself was never found in the excreta or plasma or as a tissue residue under the analytical conditions used in this study.

Most residues found in kidney and liver remained with the tissue following extraction or were retained with the macromolecule-associated fraction after filtration, suggesting binding to macromolecules (95.7 and 90.5% of the ^{14}C, respectively). The binding of desfuroylceftiofur to macromolecules is reversible; desfuroylceftiofur is excreted unchanged into the bladder, or it is conjugated with cysteine or glutathione before its excretion into the bladder (Jaglan et al., 1989). All of the metabolites in the tissue extract that were not associated with macromolecules corresponded to polar metabolites devoid of the β-lactam ring necessary for biological activity.

DFC-dimer was the most abundant metabolite in sheep urine. This metabolite is likely an artifact due to oxidation and dimerization of DFC in standing urine of pH >8 (Jaglan et al., 1989). The pH of sheep urine was >8.5. Small amounts of two DFC-conjugated metabolites were found. Polar metabolites devoid of a β-lactam ring comprised 39% of the total residues.

In plasma, DFC was conjugated to serum albumin, as has been previously found in rats (Jaglan et al., 1989). This could be expected since it is known that thiols and disulfide-containing compounds react spontaneously with endogenous disulfides and thiols (Bathala et al., 1982). A free intact β-lactam ring was not found in plasma, urine, feces, or any tissue extract.

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