Disposition of Ceftiofur Sodium in Swine following Intramuscular Treatment

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Ceftiofur displays a broad spectrum of bactericidal activity against both Gram-positive and Gram-negative bacteria including β-lactamase-producing strains. Three to five milligrams of ceftiofur per kilogram of body weight injected intramuscularly (im) on three consecutive days at 24-h intervals is recommended for bacteria-associated respiratory disease in swine. Metabolism studies in 12 swine given 5 mg of [^{14}C]ceftiofur/kg of body weight im for 3 consecutive days showed that 61.8 ± 4.7% of the dose was excreted in urine and 10.8 ± 5.1% of the dose was excreted in feces. The highest blood residues of about 15 ppm of [^{14}C]ceftiofur free acid equivalent were observed at 2 h after treatments, which declined to ~2 ppm at 24 h. Almost all of the radioactivity in the blood was found in the plasma, suggesting that the drug did not penetrate the erythrocytes. Most of the radioactivity was bound to macromolecules in the plasma and tissues. Desfuroylceftiofur glutathione disulfide was found only in the liver and 3,3′-desfuroylceftiofur disulfide (dimer) only in the urine. Desfuroylceftiofur cysteine disulfide was present in plasma, tissues, and urine. The structures of the metabolites were deduced from HPLC comparison with synthetic standards, proton NMR, and thermospray mass spectrometry. Polar metabolites are present in urine and tissues after 12 h of treatment, indicating β-lactam and cepham ring opening. Details of disposition of ceftiofur in swine are presented and compared with rats from multiple oral dosing.

Keywords: *Ceftiofur sodium; swine; metabolism; cephalosporin antibiotic*

INTRODUCTION

Ceftiofur (Figure 1) is very effective in control of Gram-positive and Gram-negative bacterial pathogens of veterinary importance both *in vivo* and *in vitro* (Yancey *et al.*, 1987). Its sodium salt, NAXCEL Sterile Solution, has been approved by the FDA for intramuscular injection to cattle, dairy cattle, swine, and day-old chickens to treat respiratory diseases (Food and Drug Administration, 1988, 1991, 1992). Like other cephalosporins, ceftiofur inhibits cell wall synthesis in bacteria, but its broad spectrum activity in part is attributed to its resistance to attack by bacterial β-lactamases due to the presence of the methoxy side chain of the imino group (Neu, 1982). Its metabolism has recently been studied in rats (Jaglan *et al.*, 1989) and dairy cattle (Jaglan *et al.*, 1992). In this paper we describe the metabolism of ceftiofur in swine (target species) following intramuscular treatment and in rats (the species chosen for toxicological evaluation) from oral doses.

EXPERIMENTAL PROCEDURES

Test Materials. Structures of the compounds described below are shown in Figure 1.

(*a*) [^{14}C]Ceftiofur sodium (**I**) labeled at the 2-position of the thiazole ring was synthesized by Chemsyn Science Laboratories, Lexena, KS. The radiochemical purity was about 97% and had a specific activity of 15 mCi/mmol.

(*b*) Unlabeled ceftiofur sodium was available from The Upjohn Co.

(*c*) [^{14}C]Desfuroylceftiofur (DFC) was typically prepared as follows: 130 μL of standard [^{14}C]ceftiofur containing 14 000 dpm (13.7 μg) in 0.1 M ammonium acetate was adjusted to pH 8.7 with 0.1 M sodium hydroxide. One hundred microliters of dithioerythritol (Sigma Chemical Co.) in water (pH 8.7) containing 400 μg was added and incubated for 20 min at 50 °C. This was charged to a prewashed (methanol followed by 0.1 M ammonium acetate, pH 7.0) C_{18} solid-phase extraction cartridge (6 cm^3). It was rinsed with 2 volumes of ammonium acetate and the DFC eluted with 1 volume of methanol. The methanol was evaporated to dryness, and the residue was taken up in an appropriate HPLC solvent for chromatography.

(*d*) [^{14}C]Desfuroylceftiofur cysteine disulfide (DCD) was also prepared. [^{14}C]Ceftiofur was processed as described under DFC. After the cartridge was rinsed with 0.1 M ammonium acetate, the cartridge was saturated with 2 mL of cystine solution (10 mg/mL in 2.1 M ammonium acetate, pH 8.7) and allowed to flow by gravity. DCD was then eluted with 2 volumes of methanol, evaporated to dryness, and taken up in an appropriate HPLC mobile phase.

(*e*) [^{14}C]Desfuroylceftiofur glutathione disulfide (DGD) was prepared in a similar manner to DCD except oxidized glutathione (Sigma) was used instead of cystine.

(*f*) [^{14}C]-3,3′-desfuroylceftiofur disulfide (dimer) was prepared by allowing [^{14}C]DFC to stand at room temperature overnight in 10% H_2O_2 solution in water. This was charged to a C_{18} cartridge and eluted with methanol as described above.

(*g*) [^{14}C]Polar C [*N*-[2-(2-amino-4-thiazolyl)-(*Z*)-2-(methoxyimino)acetyl]-2-aminoethanol], labeled as the alcohol fragment whose chemical name is given in brackets, was a gift from the Sankyo Co., Japan, to Dr. John Greenfield, The Upjohn Co.

Intramuscular Treatment of Swine. Twelve Yorkshire-Hampshire breed swine (six males and six females, each weighing about 40 kg and about 5 months old) were acclimated in metabolism cages for 3 days and were given three intramuscular injections of 5.18 ± 0.09 mg of [^{14}C]ceftiofur free acid equivalents/kg of body weight dissolved in sterile water for 3 consecutive days at 24-h intervals. Food and water were given *ad libitum*.

Sample Collection. Blood samples were obtained at various intervals posttreatment via ear vein catheter, and all animals were sacrificed at 12 h after the third dose for tissue residue determination. Feces and urine were collected every 24 h of treatment and 12 h after the last treatment.

Comparative Metabolism in Rats. Twelve rats (six females and six males) were treated orally for 4 consecutive days with 800 mg of unlabeled ceftiofur sodium. On the fifth

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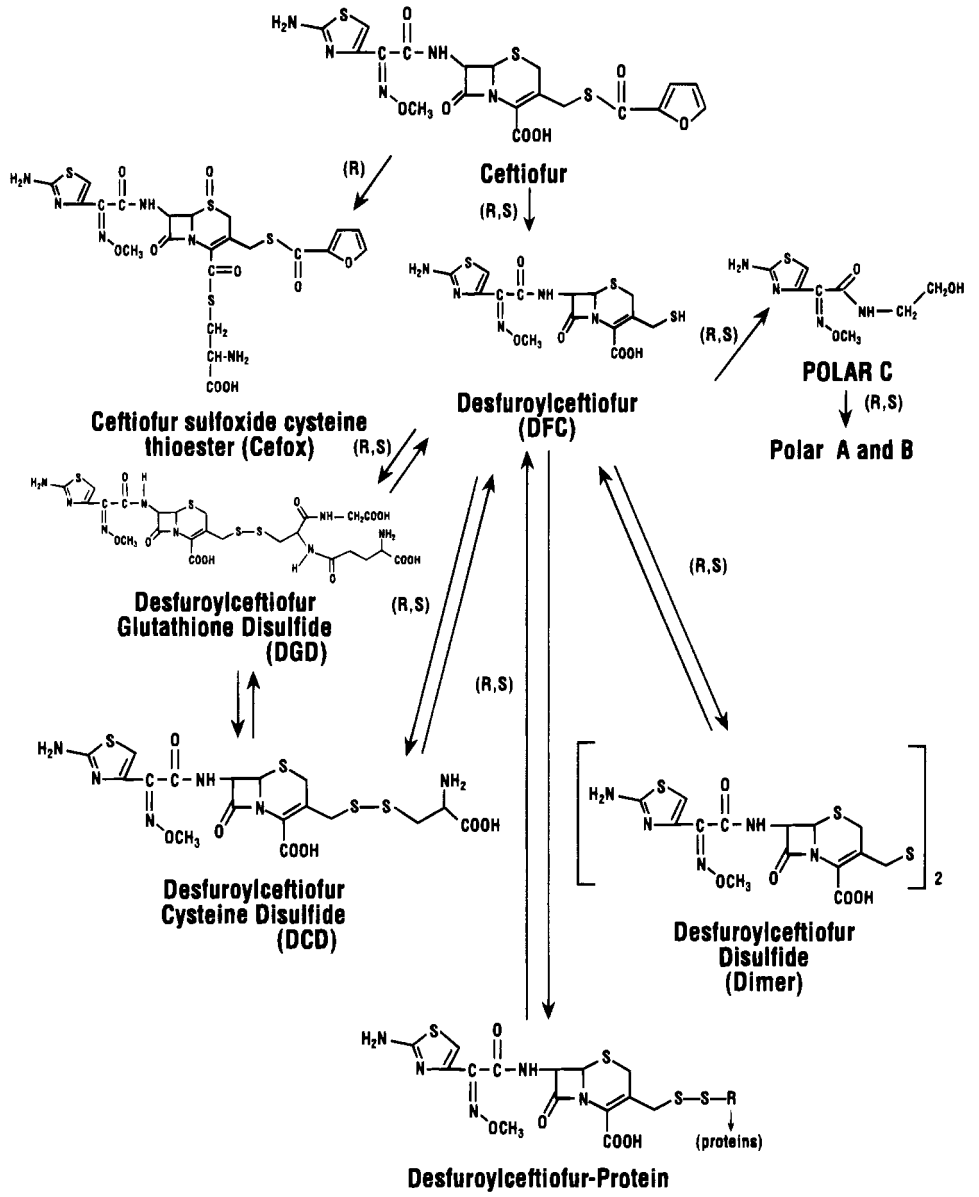


Figure 1. Proposed metabolism scheme of ceftiofur in rats (R) and swine (S).

day, the rats were dosed with 700.2 ± 2.1 mg of [^{14}C]ceftiofur free acid equivalents of ceftiofur sodium. Half of the rats were killed at 8 h and the other half at 24 h after the radioactive dose for comparing the metabolic profile with swine as described below.

Radioactive Determination. Samples of urine and plasma (about 0.5 mL) were counted in 15 mL of Instagel (Packard Instrument Co.). Blood and slurried feces (water) were weighed into Combusto cones, dried, and combusted to radioactive carbon dioxide in a biological oxidizer (Packard Instrument). Samples of tissues were ground in 2 volumes of water, and about a gram of slurry was combusted similar to feces. All liquid scintillation counting (LSC) was done with a Packard Tri-Carb liquid scintillator spectrometer, Model LC 200, using an external method of quench correction with data output to a PC disk drive system.

Metabolic Profiles. An appropriate amount of urine was diluted with 0.01 M ammonium acetate, pH 5.0, so as to obtain ~15 000 dpm/mL. The diluted urine was filtered through a 0.2 μm filter and was analyzed by HPLC (Perkin-Elmer) with diode array detection and connected to a Radiomatic Flow-One radioactive flow detector. The column was 4.6 × 220 mm RP-18 (5 μm) (Brownlee Lab). Mobile phase A was 0.01 M ammonium acetate, pH 5.0, and mobile phase B was methanol/water (60:40). A linear gradient of 25 min from 0% to 39% B was used. After each sample, the column was re-equilibrated with mobile phase A for 25 min.

Instrumental Analysis. *Proton NMR.* The metabolites DCD and DGD were analyzed by Varian XL 400 FTNMR. The metabolites were dissolved in methanol-d_4. The chemical shifts were related to TMS at 0 ppm. The resonances representing the protons were interpreted from the chemical shifts (parts per million), and the ratio of protons in each chemical grouping was established by integration.

Thermospray Mass Spectrometry. The metabolites were analyzed by thermospray interface (Finnigan) to the mass spectrometer (Finnigan 4600 quadrapole) and were analyzed at 80 °C in 30:70 methanol/0.05 M ammonium acetate. Both protonated and fragment ions were obtained.

RESULTS AND DISCUSSION

Excretion of Radioactive Dose. The excretion of radioactivity in 12 swine following three consecutive doses of 5.18 ± 0.09 mg of [^{14}C]ceftiofur free acid equivalents/kg of body weight is shown in Table 1. Most of the dose was excreted in urine and feces. Because the animals were slaughtered 12 h after the last dose, the remainder of the dose was probably still present in the GI tract. The small amounts of ^{14}C found in the

Table 1. Dose Accountability in Excreta of Swine

animal no.	total dosage (mg)	% of dose excreted urine	feces	total
89P006-02	607.31	61.55	7.80	69.35
89P014-10	677.96	65.58	9.21	74.79
89P018-03	727.50	62.48	6.54	69.02
89P030-07	542.48	64.51	26.30	90.80
89P032-07	525.47	69.52	9.77	78.89
89P034-06	558.02	54.18	11.61	65.79
89P041-08	541.71	61.21	10.11	71.32
89P046-02	544.70	59.55	9.21	68.76
89P047-08	600.62	58.92	8.87	67.79
89P043-05	548.80	16.43[a]	9.85	
89P044-04	579.77	67.07	9.19	76.26
89P052-05	585.80	55.47	10.88	66.36
av ± SD		61.82 ± 4.70	10.75 ± 5.07	72.65 ± 7.33

[a]Percent of total dosage not used in the determination of the average percent due to urination spillage of the swine.

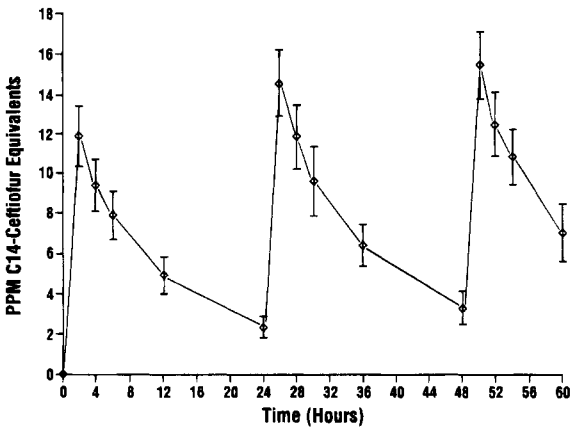


Figure 2. Total residues (PPM ^{14}C-ceftiofur equivalents) in whole blood of swine after intramuscular doses. Dosing time: 0, 24, and 48 h relative to the beginning of treatment. (Sacrificed at 12 h after last dose.)

tissues assayed suggested that carcass residues were small. Similar excretion of [^{14}C]ceftiofur in dairy cattle was observed, 62.8 ± 7.6% in urine and 35.7 ± 9.6% in feces (Jaglan *et al.*, 1992). A higher percentage of the dose was found in dairy cattle feces because feces were collected up to 5 days after the last dose compared to only 12 h in the present study.

Residues in Blood and Plasma. The maximum average blood ^{14}C residue concentrations observed at 2 h after the first, second, and third doses were 11.88 ± 1.55, 14.53 ± 1.63, and 15.44 ± 1.63 ppm as ceftiofur equivalents, respectively (Figure 2). These levels declined to 2.31 ± 0.54 and 3.30 ± 0.83 ppm at 24 h after the first and second doses, respectively. At 12 h after the third (last) dose at termination of the study, the average blood residues were 7.01 ± 1.42 ppm. The total ^{14}C residue levels in plasma were considerably higher than in whole blood. Considering that the packed cell volume (PCV) in the blood of normal swine is 38–44%, these data indicated that most of the ^{14}C residue was localized in the plasma; thus, ceftiofur residues apparently do not penetrate the erythrocytes.

Residues in Tissues. Total ^{14}C residues expressed as [^{14}C]ceftiofur free acid equivalents are given in Table 2. The highest residues were observed in the kidneys with an average of 4.47 ± 0.81 ppm, and hence this tissue is considered the target tissue. The levels are much below the safe concentration of 12 ppm established for kidneys (Food and Drug Administration, 1992). The lowest residues are observed in the muscle with an average of 0.76 ± 0.24 ppm, the tissue most consumed by the public. Total residues in other edible tissues are considerably lower than in the kidneys.

Table 2. Total Residue Levels of [^{14}C]Ceftiofur Free Acid Equivalents (Parts per Million) in Edible Tissue of Swine at 12 h after Last Dose

animal no.	sex	kidney	muscle	liver	fat	injection site	skin
89P006-02	F	4.28	0.53	1.36	1.57	2.35	2.03
89P014-10	M	3.89	0.80	1.57	1.22	3.38	1.14
89P018-03	M	3.90	0.52	1.30	2.36	3.24	2.34
89P030-07	M	4.15	0.44	1.50	1.12	2.13	0.89
89P032-07	F	5.13	0.81	1.87	2.47	2.75	0.80
89P034-06	M	4.26	0.63	1.48	1.86	2.14	0.69
89P041-08	F	3.91	1.20	1.57	0.64	2.10	1.16
89P046-02	F	4.07	0.69	1.54	1.17	3.72	1.12
89P047-08	M	6.73	1.22	1.92	1.50	6.39	1.26
89P043-05	F	4.46	0.72	1.43	1.04	2.06	1.07
89P044-04	M	4.84	0.77	1.52	1.20	1.55	0.91
89P052-05	F	4.02	0.80	1.61	1.75	2.95	1.21
mean		4.47	0.76	1.55	1.49	2.90	1.22
SD		0.81	0.24	0.18	0.54	1.28	0.52

Nature of Urinary Metabolites. Quantities of ceftiofur and ceftiofur metabolites found in urine are given in Table 3. Dimer and DCD were the principal metabolites. Identification of dimer and the mechanism of its formation due to oxidative dimerization of DFC in concentrated solution by bimolecular reaction was discussed previously (Jaglan *et al.*, 1989).

DCD as the Metabolite of Ceftiofur and Not Deacetylcefotaxime. We have previously proposed deacetylcefotaxime as a metabolite of ceftiofur (Jaglan *et al.*, 1989). Its identity was solely based on relative retention time in HPLC of the standard, and it was surmised that delactonization of the thiolactone from desfuroylceftiofur could give rise to deacetylcefotaxime. However, when this metabolite was treated with dithioerythritol, it gave rise to DFC, indicating the metabolite is a derivative of DFC. To characterize this metabolite, 10 piglets were overdosed with a single oral dose of 300 mg of [^{14}C]ceftiofur free acid/kg of body weight. To obtain enough metabolite for characterization, the metabolite from the urine was isolated by preparative HPLC and characterized by proton NMR and thermospray mass spectrometry. Figure 3 shows the thermospray mass spectrum of the metabolite. It showed a protonated molecular ion at 549 Da and its sodium adduct at 571 Da, which matched the theoretical molecular mass of DCD ($C_{17}H_{20}N_6O_7S_4$) as 548 Da. Loss of cysteine gave rise to an ion at 430 Da, which is protonated desfuroylceftiofur. The ion at 243 Da is a common hydrolytic decomposition product generated during thermospray ionization and is present in ceftiofur and all first-stage metabolites, *viz.* desfuroylceftiofur, desfuroylceftiofur disulfide, and DCD. A further loss of 42 Da from 243 Da gives rise to an ion at 201, which is also present in ceftiofur, DCD, and other metabolites. The mass spectrum is similar to that of synthetic DCD.

The NMR chemical shifts of various protons (Table 4) in the ceftiofur standard are compared with DCD isolated from piglet bladder urine. Only one aromatic proton as a singlet is observed at 7.10 ppm in DCD, showing that the other aromatic protons in the furoic acid part of ceftiofur, *viz.* a, b, and c as doublets, are absent, indicating loss of the furoyl moiety during metabolism in piglets. The protons e and f are present in both ceftiofur and DCD near 5.2 and 5.8 ppm, indicating the β-lactam ring is intact. The oxime methyl protons (g) show a similar chemical shift of about 4 ppm

Table 3. Ceftiofur Metabolite Profile in Swine Urine Samples of 12 h after Last Dose

		% of total dpm in HPLC elutes				
swine no.	retention time: metabolite:	31 min DCD	36 min DFC	42 min ceftiofur sulfoxide	47 min dimer	49 min ceftiofur
89P006-02		23.32	1.07	1.05	19.31	17.46
89P014-10		22.10		1.61	15.70	18.41
89P018-03		10.39		1.38	39.93	49.30
89P030-07		20.37	1.05	4.85	28.46	8.01
89P032-07		25.82			19.13	3.86
89P034-06		15.12			46.40	5.35
89P041-08		22.77			29.97	18.92
89P046-02		27.21	2.00		13.64	8.13
89P047-08		21.07			31.28	12.02
89P043-05		18.65		1.79	25.80	15.92
89P044-04		32.72		1.24	7.71	8.23
89P052-05		25.78			20.35	10.00
av		22.11			23.66	14.63
SD		5.80			12.81	12.07

[a]Retention time (minutes) on HPLC chromatogram.

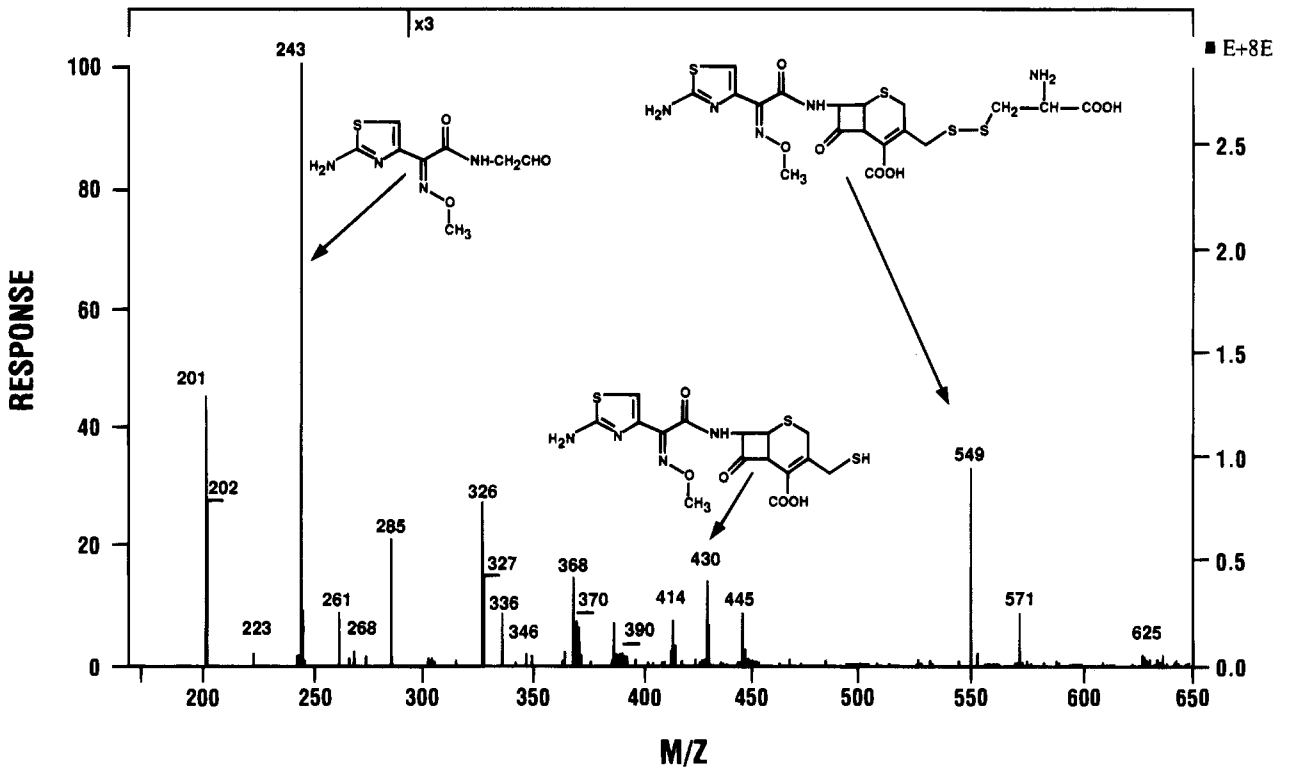


Figure 3. Thermospray mass spectrum of DCD, the metabolite of ceftiofur, and its fragmentation pattern.

in both ceftiofur and DCD and indicate the syn confirmation in the metabolite. The AB spin system of the cepham methylene (h) and C-3 side chain methylene (i) are similar in both ceftiofur and DCD, indicating the Δ1,2 isomer. The nonequivalent methylene protons and the methine protons in the cysteine part of DCD form part ABX system near 3.0–3.2 ppm and at 4.1 ppm, yielding the observed splitting pattern. Slight changes in chemical shifts of protons in ceftiofur and DCD are due to solvent D_2O and CD_3OH.

Polar C. Another polar metabolite from the urine of overdosed piglets, which had a retention time of 15 min in the HPLC system (Figure 4), gave a protonated molecular ion by thermospray LC/MS at m/z 245, indicating a molecular weight of 244. This metabolite was compared to synthetic U-80,617 ([^{14}C]polar C) by HPLC, and this polar C could not be distinguished from synthetic U-80,617 on two reversed-phase HPLC systems.

Polar C had been identified as a metabolite of another cephalosporin antibiotic which has the same side chain in the 7-position as ceftiofur. This metabolite results from cleavage of the β-lactam ring of ceftiofur followed by decarboxylation and reduction of the resulting aldehyde.

Nature of Tissue Metabolites. Among edible tissues, the kidney had the highest residue level at 12 h after the last (third) dose (Table 2) and therefore was identified as the target tissue. For analysis of the metabolite profiles in the kidney, standard methods of equilibrium dialysis and trichloroacetic acid (TCA) precipitation were used to estimate the fractions of free and macromolecule-bound ^{14}C residues with respect to the total ^{14}C residues in the kidney. By equilibrium dialysis, it was found that an average of 62.6 ± 4.6% of the total residues in the kidney were bound to proteins and 37.4 ± 4.6% were free. By the TCA precipitation,

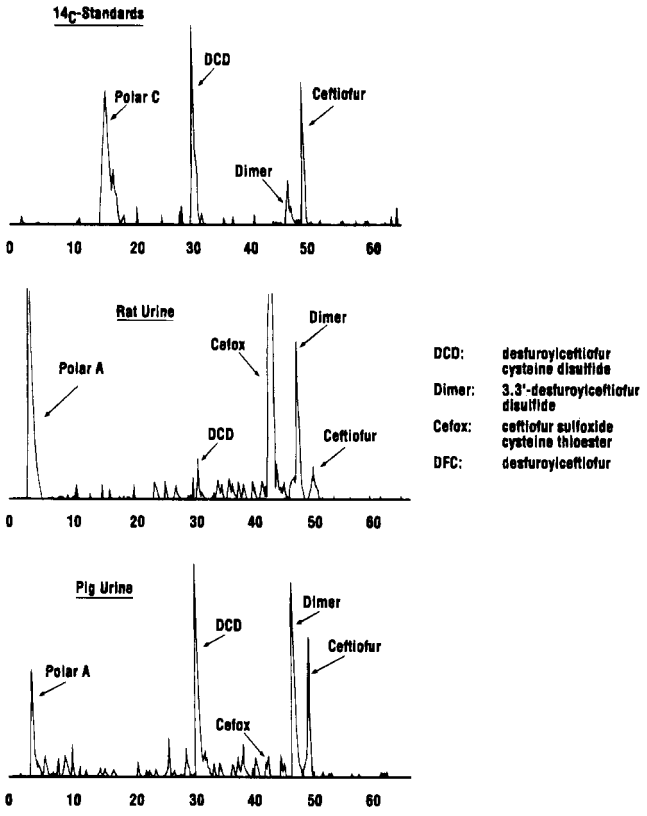
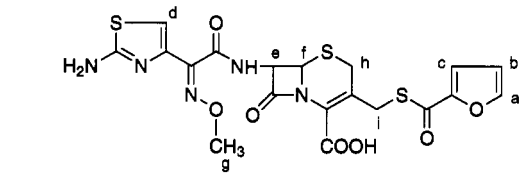
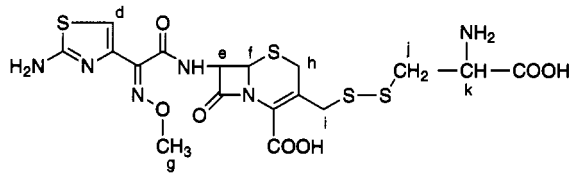


Figure 4. Typical HPLC profile of ^{14}C standard (upper), rat urine (middle), and pig urine (lower). Only ^{14}C trace is shown.

Table 4. Comparative Chemical Shifts (Parts per Million) of Ceftiofur and Metabolite DCD



ceftiofur



desfuroylceftiofur cysteine disulfide (DCD)

assignment	ceftiofur	DCD
a (aromatic doublet)	7.74	absent
b (aromatic doublet)	6.62	absent
c (aromatic doublet)	7.33	absent
d (aromatic singlet)	6.95	7.10
e (CHN)	5.78	5.75
f (CHN)	5.17	5.25
g (OCH_3)	3.97	4.02
h ($^1/_2$ CH_2AB)	3.33	3.41
h ($^1/_2$ CH_2AB)	3.64	3.83
i ($^1/_2$ CH_2AB)	3.91	3.53
i ($^1/_2$ CH_2AB)	4.20	4.15
j ($^1/_2$ CH_2AB)	absent	3.04
j ($^1/_2$ CH_2AB)	absent	3.22
k	absent	4.10
solvent	D_2O	CD_3OH

it was found that 62.1 ± 5.9% of the total residues were precipitated as protein-bound and 37.9 ± 5.9% were free.

Equilibrium dialysis is a very mild method to separate the free from bound ligands. In contrast, TCA precipitation is a very vigorous method because TCA is a very strong protein denaturant that precipitates out all proteins instantaneously and releases the noncovalently bound ligands. The results from the two different methods agreed with each other, suggesting that all of the bound residues were covalently bound to the proteins.

The free metabolite profiles in the kidney were further analyzed by HPLC. A typical HPLC chromatogram for the kidney extracts of swine showed four radioactive metabolite peaks. The metabolites were unknown polar A, unknown polar B, polar C, and desfuroylceftiofur cysteine disulfide (DCD). In relationship to the total ^{14}C in the kidney, the protein-bound metabolite accounted for 62.6 ± 4.6% (~2.80 ppm), DCD for 12.3 ± 4.1% (0.6 ± 0.2 ppm), unknown polar A for 7.6 ± 2.3% (0.3 ± 0.1 ppm), unknown polar B for 4.3 ± 3.1% (0.2 ± 0.1 ppm), and polar C for 11.3 ± 2.9% (0.5 ± 0.2 ppm).

The major metabolite in the liver was characterized as DGD on the basis of relative retention time with the [^{14}C]DGD standard.

Comparative Metabolic Profiles between Rats and Swine. Metabolic profiles of ceftiofur in the urine and kidney extracts of rats dosed orally with multiple high doses (~700 mg of [^{14}C]ceftiofur free acid equivalents/kg of body weight) were compared by HPLC systems to the urine and kidney extracts of pigs dosed intramuscularly with three consecutive doses at 5.18 ± 0.09 mg of [^{14}C]ceftiofur free acid equivalents/kg of body weight.

About 65% of the residue was bound to macromolecules and ~35% was free in the kidneys of both rats and pigs. Desfuroylceftiofur cysteine disulfide was the major metabolite in the kidneys of both rats and pigs. Metabolite polar C [*N*-[2-(2-amino-4-thiazolyl)-(*Z*)-2-(methoxyimino)acetyl]-2-aminoethanol] and unknown polar A and B were present in the kidneys of both rats and pigs.

Metabolic profiles in the urine of rats and pigs (Figure 4) contained unmetabolized ceftiofur, 3,3′-desfuroylceftiofur disulfide, and unknown polar A as significant metabolites. Desfuroylceftiofur cysteine disulfide, small amounts of unknown polar B, and desfuroylceftiofur were present in both rats and pigs. Ceftiofur sulfoxide cysteine thioester was the major metabolite in rats but was minor in pigs. This metabolite is also minor in rats from intramuscular dosing similar to that done on pigs. These data indicate that GI bacteria from oral dosing of rats probably are responsible for generation of this metabolite.

Quantitative differences in the amount of metabolites between animals as well as species are probably due to the reversibility of the disulfide metabolites and the amounts of endogenous reactants such as cysteine and glutathione and their oxidized products. Both species appear to metabolize ceftiofur in the same manner. Thus, the rat has been autoexposed to the same metabolites that man would be from the consumption of ceftiofur-treated pork, and the rat is therefore an appropriate toxicological test species. Figure 1 shows the proposed metabolism scheme of ceftiofur in swine and rats.

Conclusions. Ceftiofur sodium is a safe and effective antibiotic for swine when used according to label directions at the recommended three consecutive daily intramuscular doses of 3–5 mg of ceftiofur/kg of body

weight at 24-h intervals. Most of the dose (61.8 ± 4.7%) was excreted in urine, while 10.8 ± 5.1% of the dose was excreted in feces. The highest blood residues of about 15 ppm of [^{14}C]ceftiofur free acid equivalents were observed at 2 h after treatments, which declined to ~2 ppm at 24 h. Almost all of the radioactivity in the blood was found in the plasma, suggesting that the drug did not penetrate the erythrocytes. Most of the radioactivity was bound to macromolecules in the plasma and tissues. Desfuroylceftiofur glutathione disulfide (DGD) was found only in the liver and 3,3′-desfuroylceftiofur disulfide (dimer) only in the urine. Desfuroylceftiofur cysteine disulfide (DCD) was present in plasma, tissues, and urine. Polar metabolites are present in urine and tissues after 12 h of treatment, indicating β-lactam and cepham ring opening.

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