# Metabolism of Ceftiofur. Nature of Urinary and Plasma Metabolites in Rats and Cattle

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The metabolism of [$^{14}$C]ceftiofur following intramuscular (im) administration to cattle and oral dosing in rats produced a single metabolite, desfuroylceftiofur, which was observed in the plasma of both species. Desfuroylceftiofur existed free in the plasma of cattle but was covalently bound to plasma proteins in rats. Urinary metabolites from both species appear qualitatively similar but quantitatively different depending on the dose, route of administration, and the time interval posttreatment. Most of the urinary metabolites in rats and cattle are derivatives of desfuroylceftiofur and are probably artifacts due to the ease of its oxidation in base, lactonization in acids, and hydrolysis of lactones. An interesting metabolite ceftiofur sulfoxide cysteine is the major metabolite in the urine of rats dosed orally at or above 100 mg/kg. However, it was not present at oral doses of 7–15 mg/kg nor after im treatment of cattle and rats.

Ceftiofur (I-B, Table I) 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., 1986). Its sodium salt, NAXCEL, has recently been approved by the FDA (FDA, 1988) for the treatment of respiratory diseases of cattle. Ceftiofur is a new broad-spectrum veterinary cephalosporin. Its broad spectrum of activity is in part attributed to its resistance to attack by bacterial $\beta$-lactamases due to the presence of the methoxy side chain of the imino group (Neu, 1982). Metabolism of various cephalosporins that differ from ceftiofur only in substitution at the 3-position of the dihydrothiazine ring has been studied recently (Machinist et al., 1984; Nakayama et al., 1984; Neu and Shrinivasan, 1981; Patel and Kaplan, 1984) following intravenous and intramuscular injections in humans. These cephalosporins are not metabolized and are excreted primarily in the urine during the first 24 h posttreatment. However, cefotaxime, which has a labile side chain, is metabolized and desacetylcefotaxime is the major metabolite in rats, dogs, and man from im and iv treatments (Chamberlin et al., 1980). In this paper, we describe the metabolism of ceftiofur in cattle (target species) and rats (the species chosen for toxicological evaluation). Most of the comparison of the metabolism will deal with plasma and urinary metabolites. The nature of tissue residues and their analysis will be described in another paper.

## EXPERIMENTAL SECTION

**Test Materials.** [$^{14}$C]Ceftiofur (I-B) labeled at the 2-position of thiazole ring was synthesized by D. B. Johnson of The Upjohn Co. (Table I, I-B). Radiolabeled compound was found to be more than 98% pure, and specific activities of various lots were ~12 $\mu$Ci/mg.

**Other Compounds.** Other compounds used are described in Table I along with the approximate retention time in the two solvent systems used (see HPLC section).

**Oral Treatment of Rats.** Individual Sprague–Dawley (Charles River) rats of each sex (six males and six females), about 10 weeks old and weighing 200 g, were acclimated for 3–4 days in metabolism cages where urine could be collected separately from feces. Food and water was given ad libitum. The dose 180 mg/kg was prepared by mixing [$^{14}$C]ceftiofur sodium salt with unlabeled ceftiofur sodium to obtain a specific activity of ~5000 dpm/$\mu$g. The ceftiofur sodium was dissolved in Vehicle 122 (0.25% methylcellulose in water, The Upjohn Co.) and 0.005 g/mL of Tween 20 [poly(oxyethyl)sorbitan, monolaurate; Atlas Chemical Co.] such that each rat received 2 mL of the formulated drug. Each rat was given a single oral dose by gastric intubation and put in a metabolism cage immediately after dosing. Preliminary study as described above was also done at doses of 7, 15, and 95 mg/kg.

**Intramuscular Treatment of Rats.** Twelve rats, six male and six female, were treated im with 2, 8, or 40 mg/kg doses (two rats of each sex at each dose level) of [$^{14}$C]ceftiofur sodium. Urine of one rat of each sex at each dose level was collected in 5 mL of 0.5% trifluoroacetic acid (TFA) while the urine from the other rat was collected without any TFA for demonstration of the effect of acid during the collection of urine on the nature of the urinary metabolites.

**Sample Collection.** Urine and feces were collected at 6, 12, 24, 48, and 72 h or at other designated intervals (see above, special collection of urine from im treatment). Rats were euthanitized at various intervals posttreatment by carbon dioxide asphyxiation and total liver, kidney, and GI tract and large amounts of blood, muscle, and fat were collected. Blood was heparinized and centrifuged, and plasma was harvested.

**Cattle Urine and Plasma.** Samples of cattle plasma at 0, 0.5, 1, 2, 4, and 8 h and urine at 6, 12, and 24 h posttreatment of im dose of 2.2 mg/kg [$^{14}$C]ceftiofur sodium (activity 4000 dpm/$\mu$g) were obtained from two cattle (one male and one female).

**Preparation of Samples for Metabolic Profiles.** Urine and plasma samples of rats and cattle were diluted with 0.5% TFA, further diluted if necessary in order to obtain about 6000 dpm/mL, filtered, (0.2 $\mu$m) and analyzed by HPLC.

**HPLC System.** A Varian Vista 5560 HPLC equipped with a Varian UV detector (254 nm), Varian 8085 autosampler, HP3390A integrator hooked to a radioactive detector Flo-one Model HP (Radiomatic Instruments and Chemical Co., Inc.) LKB dual-pen recorder, Model No. 2210, and Baker Bond 300-Å-wide pore $C_8$, octyl 100 × 4.6 mm column, was used in the analysis.

**HPLC Conditions.** *System 1.* Mobile phases: A, 100% high-purity water containing 0.1% TFA; B, 90% acetonitrile containing 0.1% TFA. The gradient was run isocratic for 5 min, 16% B up to 13 min followed up to 19% B up to 43 min and 90% B up to 50 min, and then isocratic up to 55 min. The flow was 1 mL/min, and a 10-min equilibration period was allowed at the end (55 min) to permit the column to return to 100% water.

*System 2.* Mobile phases: A, 0.01 M ammonium acetate (pH 5.0); B, methanol–water (60:40). The gradient was run for 25 min from 0% B to 29% B at 1 mL/min. The column was equilibrated for 10 min after the 25-min run to return to 100% A.

**Characterization of Metabolites.** *Rat Plasma.* Rat plasma (2.5 mL) containing ~20000 dpm was diluted with 2.5 mL of pH 8.6 0.5 M Tris-HCl buffer containing 0.003 M EDTA. This was transferred to a vial and purged with nitrogen for 10 min and 200 mg of dithioerythritol added. The vial was capped and incubated for 1 h at 50 °C. The vial was cooled to room temperature, 700 mg of iodoacetamide added under $N_2$, and the solution kept in dark for 15 min. The reaction solution was then charged to $C_{18}$ cartridges (prewashed with methanol and water) and the metabolite derivative eluted with methanol. The methanol eluate was evaporated off, the residue dissolved in 3.5 mL of water

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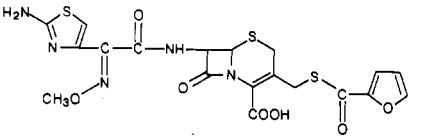
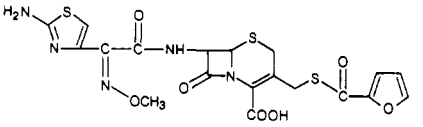
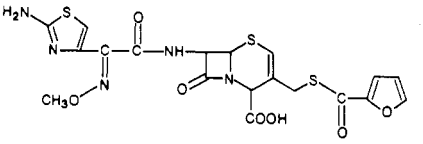
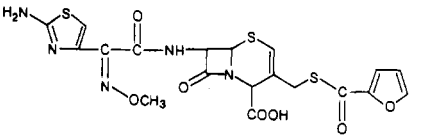
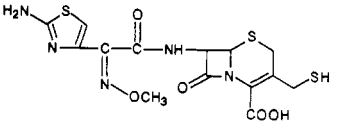
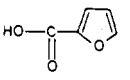
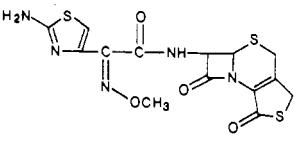
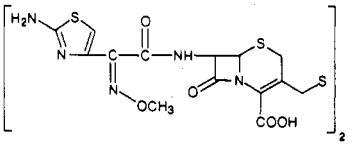
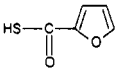
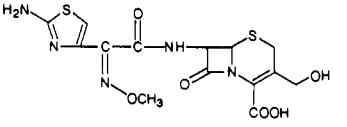
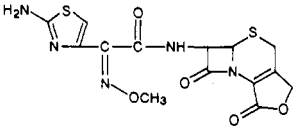
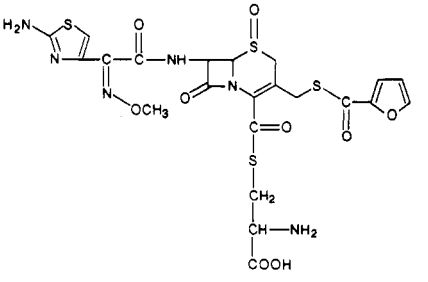
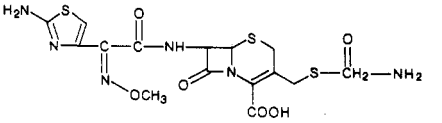
0021-8561/89/1437-1112$01.50/0 

**Table I. Number, Name, and Structures of Compounds Described in the Text and Retention Time (min) in Two HPLC Systems**

| no. | common name | structure | HPLC system[a] I | HPLC system[a] II |
|---|---|---|---|---|
| I-A | ceftiofur (anti $\Delta^3$) |  | 25 | ND[c] |
| I-B | ceftiofur (syn $\Delta^3$) |  | 28 | 38 |
| I-C | ceftiofur (anti $\Delta^2$) |  | 35 | ND |
| I-D | ceftiofur (syn $\Delta^2$) |  | 38 | ND |
| II | desfuroylceftiofur |  | 16 | 17 |
| III | furoic acid |  | 6 | ND |
| IV | desfuroylceftiofur thiolactone |  | 15 | 21 |
| V | 3,3′-desfuroylceftiofur disulfide |  | 26 | 38 |
| VI | thiofuroic acid |  | 7 | ND |
| VII | deacetylcefotaxime |  | 14 | 8 |
| VIII | deacetylcefotaxime lactone |  | 13 | 15 |
| IX | ceftiofur sulfoxide cysteine[b] |  | 22 | ND |
| X | desfuroylceftiofur acetamide |  | 15 | 15 |

[a] See text for HPLC systems. [b] Structure based on limited spectral data. [c] Not determined.

containing 0.1% TFA, and 0.5 mL analyzed by HPLC in system 1.

*Cattle Urine.* A 50-mL sample of cattle urine collected after 6 h of treatment with [$^{14}$C]ceftiofur (IB), sodium salt, at the rate of 2.2 mg/kg containing 28.9 ppm (3130 dpm/$\mu$g) was charged to 10 $C_{18}$ (6-cm$^3$) cartridges. The cartridges were prewashed with methanol and water. The radioactive metabolites were eluted with methanol. The methanol was evaporated off and the residue dissolved in 20 mL of 1% acetic acid and charged to 10 SCX (3 cm$^3$), which were prewashed with methanol followed by 1% acetic acid. The metabolites were eluted with 10 mL (per cartridge) of 0.1 M ammonium acetate. The major metabolite was further purified by collecting the peak at 7.15–7.45 min from repeated injection on HPLC using a Baker Bond wide-bore $C_8$ octyl 100 × 10 mm column with a gradient of 100% 0.5% acetic acid in water to 90% methanol for 10 min with a flow of 1 mL/min.

*Rat Urine.* The major metabolite at the retention time of 22–23 min was purified by collecting the peak from repeated injection of 500 $\mu$L of diluted urine in gradient system 1. The eluates were evaporated to dryness.

**Instrumental Analysis.** *Proton NMR.* A Varian XL 400 FT NMR was used. The metabolite (IX) and ceftiofur sodium standard were dissolved in methanol-$d_4$. Fourier transform of 20 000 pulses yielded the spectrum. The chemical shifts were related to TMS at 0 ppm. The resonances representing the protons were interpreted from the chemical shifts (ppm), and the ratio of protons in each chemical grouping was established by integration. The samples were also analyzed on a Bruker 500 FT, and two-dimensional (COSY) spectra were obtained.

*IR Spectra.* IR spectra were recorded on a Digilab Model FTS15E spectrophotometer after making KBR pellets of the metabolites.

*Thermospray Mass Spectrometry.* The metabolites were analyzed by a thermospray interface (Finnigan interface) to the mass spectrometer (Finnigan 4600 quadrupole) and were analyzed at 80 °C in 30:70 methanol–water with 0.05 M ammonium acetate. Both protonated molecular and fragment ions were obtained. High-resolution mass spectrometry was done on IX and its exact mass determined.

*Amino Acid Analysis.* The samples of cysteine hydrochloride, ceftiofur, and metabolite IX were evaporated to dryness and hydrolyzed in 6 N HCl for 24 h at 110 °C in evacuated tubes (Moore and Stein, 1963). All the samples were evaporated and reconstituted in 100 $\mu$L of pH 2.2 sodium citrate buffer. A 50-$\mu$L portion of each sample was oxidized with 30% $H_2O_2$ for 2 h and evaporated. The samples were reconstituted in 50 $\mu$L of water. A 20-$\mu$L portion of each sample after HCl hydrolysis as well as after oxidation was analyzed on a Dionex D505 amino acid analyzer. Peak areas were calculated and converted to nanomoles from a standard curve. Based on the recovery of cystoic acid and glycine from cysteine hydrochloride and ceftiofur, respectively, the concentration of metabolite IX was calculated and compared with the $^{14}$C content of the metabolite.

## RESULTS AND DISCUSSION

**Excretion of Radioactive Dose.** Excretion studies from im treatment of rats with 2, 8, and 40 mg/kg [$^{14}$C]-ceftiofur sodium showed that ~60% of the dose was excreted in the urine in the first 12 h of treatment and about 30% was excreted through bile in the GI tract and feces. These data were similar to cattle treated with 2.2 mg/kg [$^{14}$C]ceftiofur sodium.

The bulk of the radioactivity from a single oral dose of [$^{14}$C]ceftiofur sodium in the rats from 7 to 95 mg/kg was excreted in the feces in the first 48 h posttreatment, and only about 5% was excreted in urine. When the rats were treated with a single oral dose of 180 mg/kg [$^{14}$C]ceftiofur sodium, about 1% of the dose was absorbed and excreted in urine in the first 12 h of treatment. Most of the radioactivity was excreted in feces.

**Nature of Plasma Metabolites.** The concentration of radioactivity in the cattle plasma peaked at ~6 ppm at about 1 h postdosing with 2.2 mg/kg [$^{14}$C]ceftiofur, sodium salt, and a single metabolite, II (Figure 1), was observed.

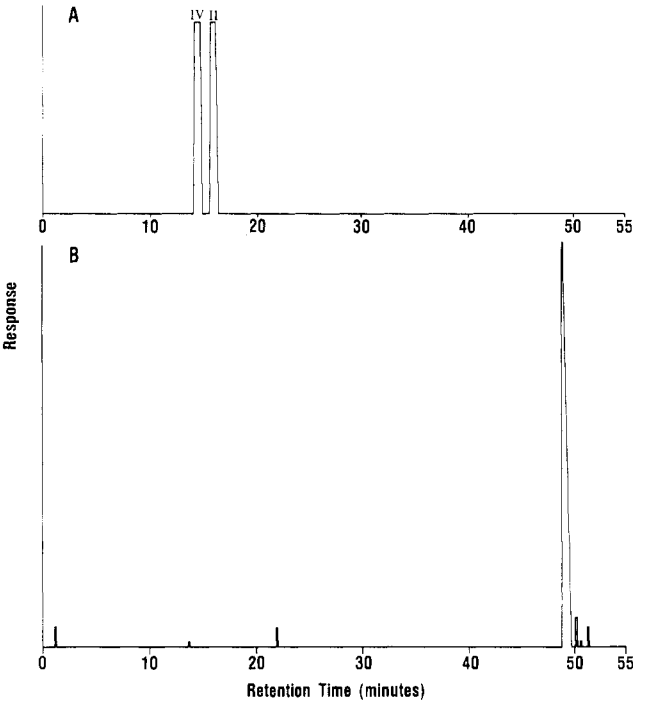



**Figure 1.** HPLC of (A) cattle plasma and (B) rat plasma following im treatment with ceftiofur (only $^{14}$C trace is shown).

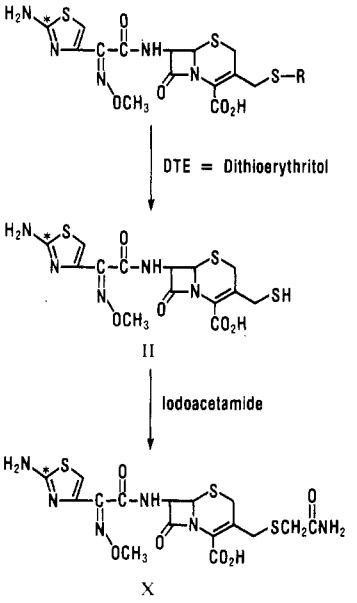



**Figure 2.** Scheme for reduction of rat plasma bound radioactivity and its derivatization.

In HPLC system 1, II lactonizes to IV and this acid-catalyzed conversion is complete in 1 h. The concentration of radioactivity in the rat plasma from im treatment with 2 mg/kg ceftiofur sodium and from a single oral dose of 180 mg/kg [$^{14}$C]ceftiofur sodium was about 0.7 ppm after 12 h of treatment. A single metabolite from both im and oral treatment was found at the retention time of ~50 min and was desfuroylceftiofur bound to protein (Figure 1). This protein has tentatively been shown to be acidic with an isoelectric point of 4.6 and a molecular weight of 59 000. Treatment of rat plasma with dithioerythritol (DTE) and derivatization with iodoacetamide (Figure 2) gave rise to X, identified as desfuroylceftiofur acetamide from the comparative retention time in two HPLC systems and to synthetic standard X. The identity of II as a single metabolite in the plasma of the cattle as free and in rats as

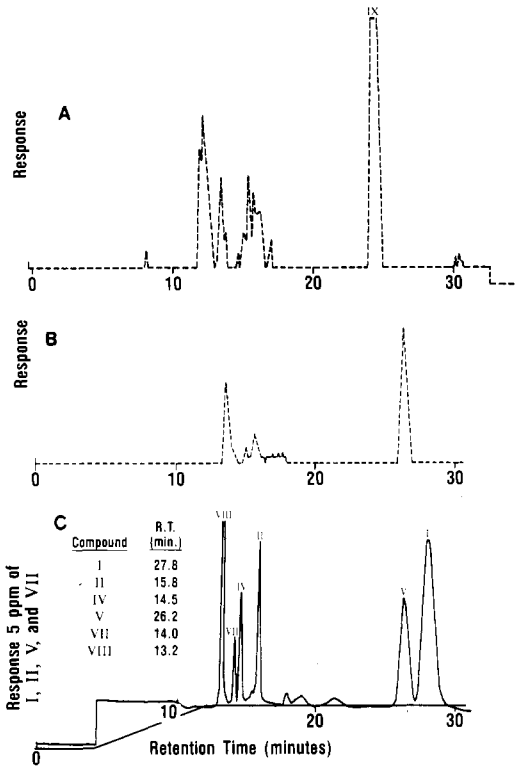



**Figure 3.** HPLC of (A) rat urine from oral dose, (b) cattle urine from im dose following [$^{14}$C]ceftiofur treatment, and (c) synthetic standards in system 1 (only $^{14}$C trace is shown, ---).

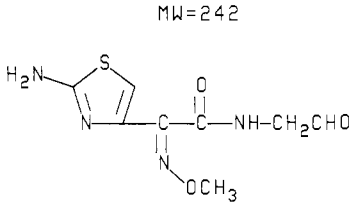
bound to protein indicates that the metabolism of ceftiofur in both species is similar.

**Nature of Urinary Metabolites.** The HPLC of rat urine collected from an oral dose of 180 mg/kg and cattle urine following im treatment of ~2.2 mg/kg ceftiofur sodium was similar (Figure 3), and metabolites II/IV and VII/VIII were present. IV and VIII are probably artifacts because II and VII lactonize under acidic conditions in HPLC system 1. The only difference was that rat urine contained trace amounts of unchanged I where no trace of I was found in cattle urine. The presence of IX in rat urine from oral doses, which was not present in rat or cattle urine from im treatment, is dose related and discussed below. V as a major metabolite in cattle urine is also discussed below.

**Effect of Dose on the Nature of Metabolites in Urine of Rats.** Most of the radioactivity excreted in the urine from the 7–15 mg/kg oral dose contained polar metabolites eluting from the HPLC in the first 5 min (Figure 4). However, at higher doses of 95 and 180 mg/kg, there are first-stage metabolites II (IV) and VII (VIII) and metabolite IX, which was not present in the cattle (see Figure 3 for metabolite number and relative retention time) and rat urine from im treatment nor from oral doses of 7–15 mg/kg. These data suggest that IX is formed in the gut probably by the action of GI tract organisms.

**Characterization of V.** The major metabolite in the cattle urine following im administration of 2.2 mg/kg [$^{14}$C]ceftiofur sodium dose was purified on solid-phase extraction columns, $C_{18}$ Bond Elut and SCX Bond Elut. The metabolite was further purified by preparative HPLC using system I and collection of the radioactive peak at 26 min from repeated injections. The retention time in HPLC and the IR spectra of the metabolite were found similar to the synthetic standard (V).

Thermospray LC/MS (Figure 5) shows the protonated molecular ion at 857 Da and has a characteristic ion at 243 Da. The ion at 243 is present in I, II, IV, IX, and X and is indicative of the cephalosporin ring connected to the thiazole ring of the molecule. The structure of aldehyde (MW 242) is shown:



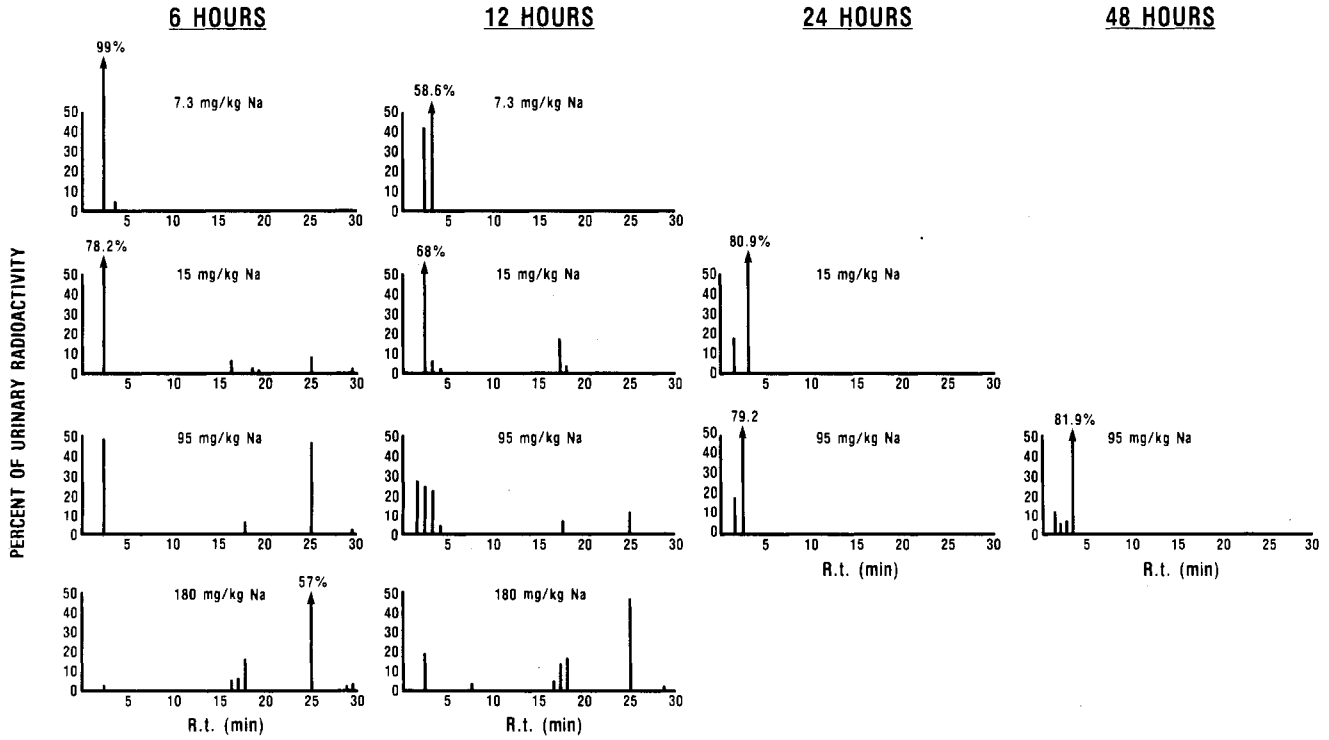




**Figure 4.** Schematic HPLC profile of radioactivity in rat urine from single doses of [$^{14}$C]ceftiofur.

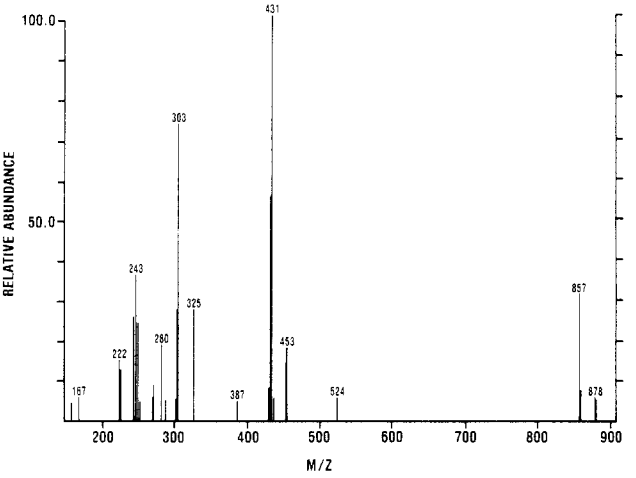



**Figure 5.** Thermospray mass spectra of the major metabolite in cattle urine following im treatment with [$^{14}$C]ceftiofur.

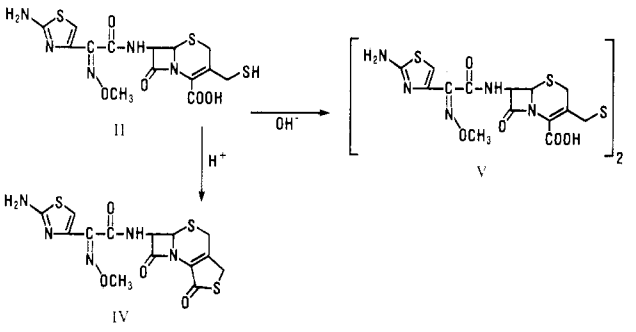



**Figure 6.** Scheme for alteration of II to IV and V under acidic and basic conditions.

**Table II. Percentages of Various Metabolites of Ceftiofur Found in the Urine of Rats Treated with an Intramuscular Dose of [$^{14}$C]Ceftiofur Sodium**

| dose rate, mg/kg | metabolites[a] I A | I B | V A | V B | II/IV A | II/IV B |
|---|---|---|---|---|---|---|
| 2 | 17.7 | 7.6 | 2.4 | 15.3 | 70.0 | 44.5 |
| 8 | 7.6 | 3.6 | 25.3 | 45.0 | 61.9 | 43.4 |
| 40 | 15.4 | 14.9 | 9.7 | 56.1 | 73.3 | 27.8 |

[a] A = urine collected in 0.5% TFA; B = urine collected without TFA.

Probably, the ion at 243 is created in solution during the hot conditions of thermospray. It was suspected that V may be an artifact since it was not observed in the plasma or tissues. Its formation in urine from II by a bimolecular reaction is suspected because II is present in urine in concentrated form. Moreover, the pH of cattle urine was found to be 8.8–9.2 probably due to contamination by feces. At pH >7 oxidative dimerization of the free sulfhydryl group of II to V is most likely (Figure 6) whereas under acid conditions II lactonizes to IV.

In order to confirm the above hypothesis, urine from rats treated with im doses of 2, 8, and 40 mg/kg [$^{14}$C]ceftiofur sodium was collected with or without 0.5% TFA because in acid conditions II will lactonize to IV, which cannot dimerize to V. Table II shows that V is present in significantly higher concentration in urine collected without TFA especially at 40 mg/kg dose. This is expected since a high concentration of II will have a high probability of dimerization by the bimolecular reaction. The data in Table II also shows that II was present in high concentration in urine collected in TFA, indicating inhibition of dimerization.

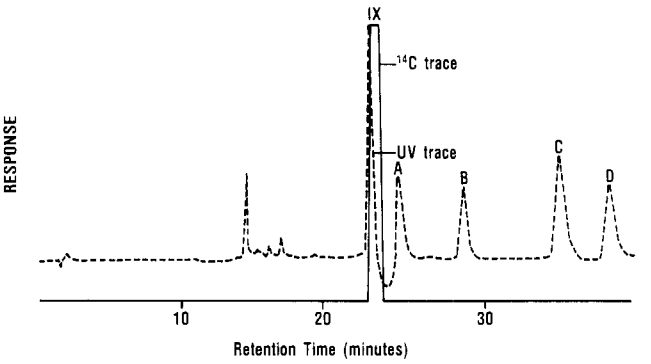



**Figure 7.** HPLC of A ($\Delta^3$-anti), B ($\Delta^3$-syn), C ($\Delta^2$-anti), and D ($\Delta^2$-syn) isomers of ceftiofur and metabolite IX: —, $^{14}$C trace; - - -, UV trace.

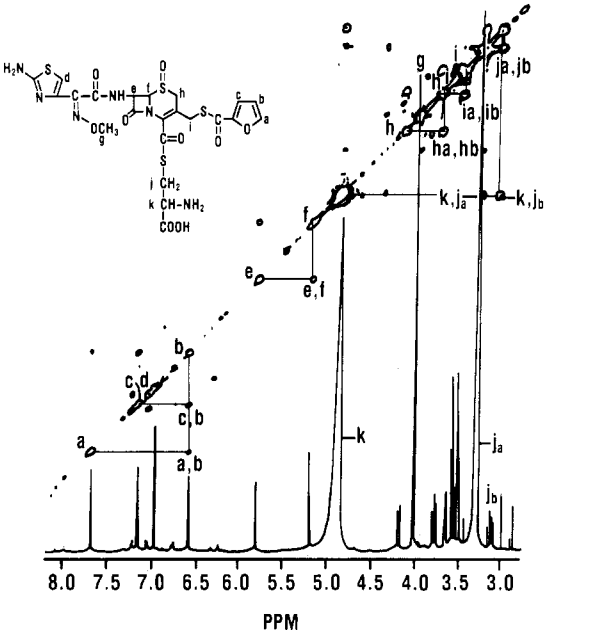



**Figure 8.** 2D proton NMR of the major urinary metabolite of ceftiofur in rats from oral dose.

**Characterization of Compound IX.** The major metabolite of ceftiofur in the urine of rats dosed orally eluted from the HPLC column (Figures 3 and 4) before I and V and was considered polar and assumed to be either the ceftiofur with the $\beta$-lactam ring open or some polar derivatve of II that was the only metabolite of I in plasma. Early NMR data indicated it was ceftiofur with all the three furoic acid protons at identical chemical shifts. The IR spectrum gave characteristic C=O ($\beta$-lactam) absorption at 1769 cm$^{-1}$, indicating the $\beta$-lactam ring was intact making both the above assumptions untenable. Previous studies with insecticide methomyl have shown that *syn*-methomyl isomerized to *anti*-methomyl in the GI tract of rats (Huhtanen and Dorough, 1976). It was suspected that ceftiofur, which has syn configuration (I-B), isomerized to *anti*-ceftiofur (I-A). In addition, isomerization of $\Delta^3$- to $\Delta^2$-ceftiofur is also likely. However, the HPLC analysis of $\Delta^3$-*syn*-ceftiofur (I-B), $\Delta^3$-*anti*-ceftiofur (A), $\Delta^2$-*syn*-ceftiofur (D), $\Delta^2$-*anti*-ceftiofur (C), and radioactive metabolite IX (Figure 7) demonstrated that this metabolite was more polar and was not an isomerization product.

Close reexamination of the NMR spectrum of the metabolite and NMR spectrum expansion revealed that metabolite protons (h) have a larger coupling constant (Figure 8) of 18 Hz as compared to 15 Hz in ceftiofur, indicating that the adjacent sulfur atom may be oxidized.

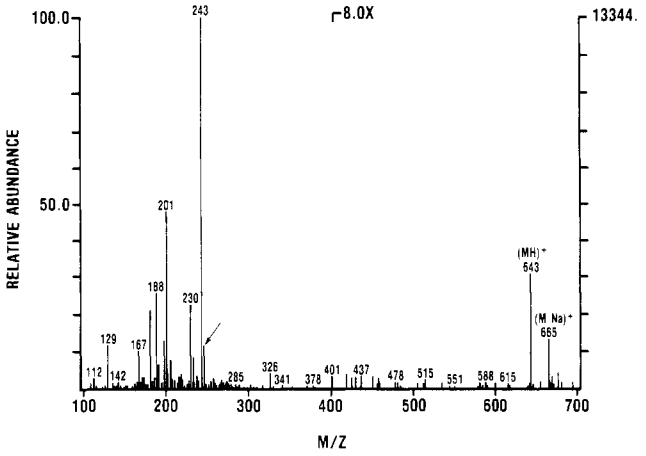



**Figure 9.** Thermospray mass spectra of the major urinary metabolite of ceftiofur in rats from oral dose.

From the 2D proton correlation spectrum (COSY) (Figure 8) proton couplings were readily determined by the analysis of cross peaks. From this spectrum, the h and i AB pattern of protons as well as the ABX pattern of j and k protons is readily apparent.

Thermospray mass spectra of IX showed $MH^+$ and $MNa^+$ at 643 and 665 Da. This indicated that the molecular mass is 642 Da, 119 amu higher than that of ceftiofur (Figure 9). The characteristic ion at 243 Da was also observed. The presence of the furoyl moiety is confirmed from ions at 112 and 129 Da for furoic acid and thiofuroic acid, respectively. The latter is a hydrolysis product formed in the thermospray source. Decomposition of thiofuroic acid in the source can also yield furoic acid. The presence of the furoic acid part of the molecule is consistent with the NMR data.

The exact mass measurement showed that the metabolite has 642.033 95 amu, and thus the ion at 642.0331 is consistent with the empirical formula and the structure (Figure 8).

Amino acid analyses of cysteine hydrochloride, ceftiofur, and metabolite IX were done after acid hydrolysis and after acid hydrolysis plus oxidation. Glycine values were compared from acid hydrolysis, and cystoic acid values were used from oxidation because acid hydrolysis alone does not convert cysteine to cystoic acid. Calculation based on the glycine and cystoic acid content of metabolite IX and comparing the values of glycine in ceftiofur and cystoic acid in cysteine hydrochloride showed the concentration of the metabolite was similar to the $^{14}C$ content of the sample.

## CONCLUSIONS

The metabolism scheme of I in rats and cattle is shown in Figure 10. I is metabolized to II and furoic acid in cattle and rats. [Further metabolism of furoic acid will generate metabolites found in the normal urine of humans (Mrochek and Rainey, 1972).] II is present as the sole metabolite in the plasma of rats and cattle. In cattle plasma II is present free, but in rat plasma it is bound to proteins from both im and oral treatments. The presence of a single metabolite, II, in the plasma of both species shows that fission of the thioester bond takes place but not the akyl–sulfur bond, contrary to cephalothin in which both alkyl–oxygen and acyl–oxygen bonds were broken (Indelicato et al., 1985). The thioester bond was possibly cleaved by plasma or hepatic esterases.

Lactonization of II and VII to IV and VIII, respectively, was desirable to stabilize the metabolites, and hence the samples were stored and analyzed in trifluoroacetic acid

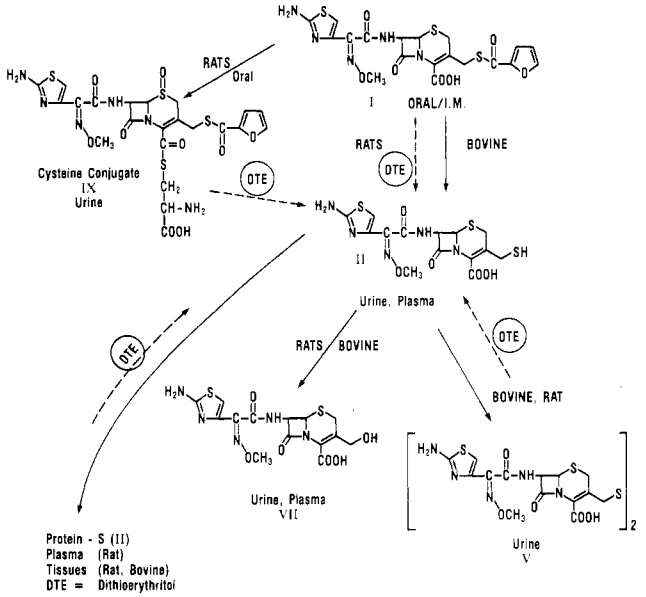



**Figure 10.** Metabolism scheme of I in rats and cattle.

containing medium. Metabolite V is likely an artifact due to oxidation and dimerization of II in standing urine of pH >8. The inhibition of the formation of V was demonstrated by collection of urine in acidic media.

The presence of VII, which could undoubtedly arise by oxidative desulfurization, may also be an artifact due to delactonization of IV to II and VII. Its identity is solely based on the relative retention time in HPLC of standard.

The metabolism of I to IX seen only in high oral doses suggests that GI bacteria and/or medium is required. Most of the naturally occurring thioester bonds are labile and short-lived, and this may be the reason that we were not able to observe IX at low dose levels.

The presence of II bound in plasma and tissues can be rationalized if we consider that thiol- and disulfide-containing compounds react spontaneously and often rapidly with endogenous disulfides and thiols, respectively (Hanzlik, 1984). For example, captopril, a thiol-containing drug, has been shown to participate in disulfide exchange reactions (Bathala et al., 1982).

Metabolism of ceftiofur in other animals will be described in the next paper.

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**Registry No.** I-A, 120882-20-4; I-B, 80370-57-6; I-C, 120882-21-5; I-D, 120962-17-6; II, 120882-22-6; III, 26447-28-9; IV, 120882-23-7; V, 120905-00-2; VI, 4741-45-1; VII, 66340-28-1; VIII, 66340-33-8; IX, 120882-24-8; X, 120882-25-9.

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# Structure–Bioactivity Relationships of Salannin as an Antifeedant against the Colorado Potato Beetle (*Leptinotarsa decemlineata*)

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Fourteen derivatives of salannin were prepared and bioassayed for antifeedant activity against larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*). Several of the derivatives, including 3-*O*-methyl-3-deacetyl-2′,3′,20,21,22,23-hexahydrosalannin and 3-deacetoxy-2′,3′,20,21,22,23-hexahydrosalannin, were over 40-fold more active than salannin as Colorado potato beetle antifeedants. Changes in the antifeedant activity were observed with chemical modification at four points of the salannin molecule: (1) hydrogenation of the furan ring, (2) replacement of the acetoxyl group, (3) modification of the tigloyl group, and (4) saponification of the methyl ester.

Salannin ($C_{34}H_{44}O_9$) is a limonoid of the tetranortriterpenoid type found to occur in at least four species of plants in the Meliaceae, including *Azadirachta indica* A. Juss. (neem) (Henderson et al., 1964, 1968), *Melia azedarach* L. (chinaberry) (Srivastava, 1986), *Melia dubia* Cav. (de Silva et al., 1969), and *Melia volkensii* Gurke (Rajab et al., 1988). The biological effects of salannin include insect antifeedant or feeding deterrency activity against *Musca domestica* L. (house fly) (Warthen et al., 1978), *Acalymma vittatum* F. (striped cucumber beetle), Diabrotica *undecimpunctata howardi* Barber (spotted cucumber beetle) (Reed et al., 1982), *Spodoptera littoralis* Boisd. (Egyptian cotton leafworm), *Earias insulana* Boisd. (spiny bollworm) (Meisner et al., 1981), *Aonidiella aurantii* Maskell (California red scale), and *Locusta* sp. (locust) (Warthen, 1979).

Although the insect antifeedant activity of salannin is well documented, little is known about its structure–bioactivity relationships. In this paper, we report on the preparation of 14 derivatives of salannin and their antifeedant activity against the agricultural pest insect *Leptinotarsa decemlineata* (Say) (Colorado potato beetle).

## MATERIALS AND METHODS

**Materials.** Solvents used for high-performance liquid chromatography (HPLC) were of HPLC grade. Other chemicals were of reagent grade or better and were used without further purification unless noted otherwise.

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**Bioassay.** Compounds were examined for antifeedant activity by leaf disk choice bioassays. Third-instar (colony reared) larvae of *L. decemlineata* were used as the test organism.

Leaves of uniform size and thickness were taken from potato plants (Russet Burbank variety) grown in a greenhouse. Disks (1 cm$^2$) were punched out from the leaves, randomized, and arranged (6 disks/cup) in a circle sandwiched between moistened filter paper (Gelman) inside plastic cups (52 × 36 mm). Only the upper surfaces of the disks were exposed. The upper surfaces of alternating disks were treated with either 25 μL of acetone (control) or between 1 and 400 μg of a test substance dissolved in 25 μL of acetone. Newly molted third-instar *L. decemlineata*, reared from hatching on Russet Burbank leaves and weighing between 35 and 40 mg, were placed 1 larva/cup at ca. 27 °C in continuous light. The disks were examined visually every 2 h until >95% of the control disks were eaten. The $PC_{95}$ value, the minimal protective concentration (micrograms/disk) at which >95% of the control disks, while <5% of the treated disks, were eaten in the choice bioassay, was determined for each test substance from 15–20 replicates/concentration. The $PC_{50}$ value, the minimal protective concentration (micrograms/disk) at which >50% of the control disks, while <5% of the treated disks, were eaten in the choice bioassay, was determined for certain test substances from 15–20 replicates/concentration.

**High-Performance Liquid Chromatography.** Preparative HPLC was carried out with a Micromeritics Model 750 solvent delivery system equipped with a Negretti and Zambra injector, a Micromeritics Model 787 variable-wavelength UV/visible detector, a Hewlett-Packard 3388A integrator/recorder, and a Gilson Model 201 fraction collector. Chromatography was accomplished with either a normal-phase Alltech Associates silica gel (10-μm particle size) stainless steel column (25 × 1.0 cm (i.d.)), protected with an Alltech Associates stainless steel guard column (5.0 × 0.46 cm (i.d.)) packed with Alltech Associates pellicular silica gel, or

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