METABOLISM OF TRITIUM- AND CARBON-14-LABELED TIAMULIN IN DOGS, RATS, AND PIGS

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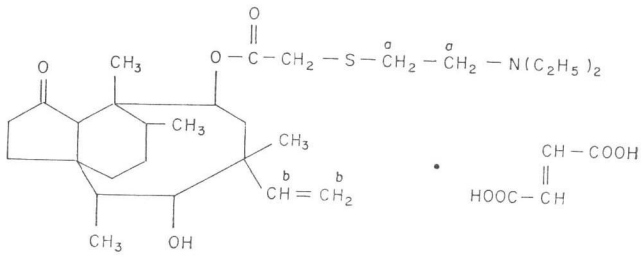
The metabolism of tiamulin hydrogen fumarate, labeled with ^{3}H, ^{14}C, or both, was studied in dogs, rats, and weanling pigs. After a dose of radiolabeled tiamulin, all three species excreted more radioactivity in feces (*via* bile) than in urine. Dogs absorbed 86% of a single oral dose of tiamulin-^{3}H, and the disposition of the compound was similar after a single or multiple dosage regimen. The ratio of antimicrobial activity to total radioactivity in dog plasma was only about 0.25, and was still less in dog urine. After dosing with tiamulin-^{14}C, rats and pigs excreted at least 1% of the dose as $^{14}CO_2$ in expired air. In dual-labeled studies, pigs excreted less total ^{14}C than ^{3}H and had greater residues of ^{14}C than ^{3}H in edible tissues, blood, and plasma. After the administration of tiamulin-^{14}C to pigs, radioactivity was incorporated into liver glycogen, indicating metabolic cleavage of the side chain of tiamulin. Tiamulin-^{3}H is the isotopically-labeled compound of choice for studying metabolism and tissue residues in animals.

Tiamulin (Fig. 1) is a diterpenoid antimicrobial agent produced semisynthetically from pleuromutilin, a product of fermentation from the Basidiomycete genus *Pleurotus*. Details of the synthesis, chemical properties, and antimicrobial activity of tiamulin and related derivatives have been published[1~4]. Clinical trials of the hydrogen fumarate salt of tiamulin have shown that it is effective against infections of mycoplasma in poultry and swine[5,6], swine dysentery[7], and enzootic pneumonia of calves and swine[8].

Fig. 1. Structure of tiamulin hydrogen fumarate.
The letter "*a*" denotes the location of ^{14}C, while the letter "*b*" denotes the location of ^{3}H.



The present report describes the results of metabolic studies using tritium- and carbon-14-labeled tiamulin in rats and dogs, species used in toxicologic investigations, and in weanling pigs, a target species. Preliminary reports of these studies have been published[9~11].

Materials and Methods

Specifications of radiolabeled tiamulin preparations

Tiamulin-^{3}H (98.4% radiochemically pure) was synthesized by Sandoz Forschungsinstitut. It was converted to the hydrogen fumarate salt and administered to dogs and pigs at a specific activity of 2.1 μCi/mg. Tiamulin-$^{3}H/^{14}C$ (98.4~98.9% radiochemically pure) was synthesized by The Squibb Institute for Medical Research. It was also converted to the hydrogen fumarate salt and administered to rats at a specific activity of 1.35 μCi/mg and to pigs at a specific activity of 0.70 μCi/mg.

Tiamulin-$^{3}H/^{14}C$ was prepared by combining the ^{3}H- and ^{14}C-labeled compounds and was administered to pigs as the hydrogen fumarate salt at a specific activity for ^{3}H of 2.1 μCi/mg and a specific activity for ^{14}C of 0.70 μCi/mg.

Studies in dogs

1. Single dose: Two groups of purebred beagles, one of each sex per group (9.0~9.6 kg), were fasted overnight and given either 3 mg/kg of tiamulin-^{3}H intravenously or 10 mg/kg in aqueous solution by gavage. During the 10-day test, blood was collected frequently and excreta were collected daily.

2. Multiple doses: Four purebred beagles, two of each sex (8.7~9.3 kg), were fasted overnight and given five consecutive daily (9: 00 a.m.) 10-mg/kg doses of tiamulin-^{3}H in aqueous solution by gavage. All doses administered contained tiamulin-^{3}H. Blood was withdrawn 4 hours after each of the first four doses, frequently after the fifth dose, and once daily thereafter. Urine and feces were collected daily during the dosing period and for an additional 10 days.

3. Anesthetized bile-duct cannulated dogs: A purebred male beagle (8.7 kg) was anesthetized with pentobarbital (30 mg/kg, i.v.). A buffered solution of mannitol and pentobarbital was infused into the radial vein during the 8-hour experiment at the rate of 3 ml/min. A catheter was inserted into the urinary bladder and the common bile duct was cannulated. The dog was given a single 2.6-mg/kg intravenous dose of tiamulin-^{3}H, and urine and bile were collected hourly. Samples of urine (15 ml) were adjusted to a pH of 8 and extracted three times with 20 ml of ethyl acetate. The extracts were combined, evaporated to dryness, and dissolved in 0.2 ml of ethanol. This procedure extracted an average of 66.5% of the ^{3}H present in urine and 98~102% of tiamulin-^{3}H added to normal dog urine. Extracts of urine (0.05 ml) and unextracted bile (0.05 ml) were chromatographed on silica gel thin-layer plates (Analtec Uniplate GF) developed in the solvent system: *n*-butyl acetate - methanol - 28% ammonia (100: 5: 1). Radioactivity was detected on the plates by scraping 1-cm sections into vials containing scintillation fluid and counting. Duplicate plates were run for each sample and one of them was subjected to bioautography, using *Sarcina lutea* ATCC 9341 as the test organism.

Studies in rats

Four fasted rats (albino Sprague-Dawley; Charles River CD), two of each sex (215~258 g), were given 30-mg/kg doses of tiamulin-^{14}C in aqueous solution by gavage, and the excretion of radioactivity was determined in urine, feces, and expired air. Rats were housed in plastic metabolic cages that permitted the separate collection of urine and feces. To measure the $^{14}CO_2$ evolved, each cage was enclosed in a plastic bag continuously purged with air. $^{14}CO_2$ in expired air was trapped with a solution of monoethanolamine and methylcellosolve (1: 3).

Studies in pigs

Four weanling pigs, Landrace York-Cross SPF Herd, (initial weights of 13~16 kg), two of each sex, were given tiamulin-$^{3}H/^{14}C$ in aqueous solution by gavage for 5 consecutive days (5.5 mg/kg at 9: 30 a.m. and at 3: 30 p.m.). All doses administered contained tiamulin-$^{3}H/^{14}C$. Blood was withdrawn 4 hours and 10 days after the ninth dose; excreta were collected daily after each dose. Ten days after the ninth dose, the pigs were sacrificed by the injection of pentobarbital (30 mg/kg, i.v.) followed by exsanguination, and samples of thigh muscle, omental fat, kidneys, and liver were collected, as well as plasma.

Experiments were also conducted in weanling pigs (purebred Landschwein; 11~22 kg) in which 10-mg/kg per day of either tiamulin-^{3}H or tiamulin-$^{3}H/^{14}C$ was administered orally for 10 days in the feed in equally divided doses of 5 mg/kg at 8: 00 a.m. and 3: 30 p.m. Residual radioactivity was determined in the edible tissues, blood, plasma and bile of these pigs at selected times after the end of the dosing period. Glycogen was extracted from the livers of two pigs (12 and 19 kg; one of each sex) treated with tiamulin-$^{3}H/^{14}C$ in the feed as described above. One pig was sacrificed 10 days after the end of treatment and the other after 25 days. Glycogen was extracted from liver homogenates by the method of MORDOH *et al.*[12] (overall recovery of 85~90%), and determined by both the anthrone method[13] and enzymatically[14].

To collect expired air from weanling pigs (Landrace York-Cross SPF Herd), two females (10.6~12.5 kg) were separately housed in small dog cages, which were surrounded by plastic bags. The pigs were given single 11-mg/kg doses of tiamulin-^{14}C in aqueous solution by gavage (120 μCi), and expired air was collected for 10 hours by the same basic procedure used for rats, but modified for pigs to allow for a greater flow of air and a more frequent changing of the CO_2-absorbing traps.

Tritiated water in urine of dogs, rats, and pigs

The presence of tritiated water in urine was determined in ancillary experiments in the dog, rat, and pig. Urine was collected by catheter from a male dog during the first hour after the intravenous administration of a 2.6-mg/kg dose of tiamulin-^{3}H. The sample of urine (5 ml) was diluted to 15 ml with water and distilled. A portion of the distillate was counted for the presence of radioactivity.

Urine was collected daily for 11 days from four male rats (200~250 g) that had been dosed orally by gavage with 50 mg/kg of tiamulin-^{3}H. Portions of the urine, pooled for each time, were lyophilized and samples of the volatile fractions were counted.

Urine was collected from pigs that had been dosed orally in the feed (10 mg/kg per day for 10 days) with either tiamulin-^{3}H (two males and two females) or tiamulin-^{3}H/^{14}C (one male and one female). Samples of urine were collected at intervals for as long as 25 days, and pooled separately by sex for each collection period. A portion of each sample was lyophilized and a sample of each volatile fraction was counted.

Analytical procedures

Samples of plasma (0.5~1 ml), blood (0.5~1 ml), urine (1 ml), and bile (0.1 ml) were added to combustion cones, and any volatiles (including tritiated water) were allowed to evaporate at room temperature for at least 24 hours. Samples of feces were homogenized with two to three volumes of methanol. About 1 g of fecal homogenate was weighed into a tared combustion cone and allowed to dry. Samples of rat carcass were ground in a meat grinder and 1 g was weighed into a tared combustion cone and allowed to dry. All samples of tissue were first cut into small pieces with scissors. For muscle and kidney, about 1 g was weighed into tared combustion cones and allowed to dry. Samples of fat (0.2 g) and liver (1 g) were also weighed into tared combustion cones that contained a layer of about 200 mg of cellulose powder; another 200 mg of cellulose powder was placed on top of the sample of fat, and it was allowed to dry. All samples of tissue were analyzed in triplicate. Samples of blood and plasma were analyzed in duplicate, if enough was available. All samples were combusted in a Model 306 Packard Oxidizer, and counted with Monophase 40® scintillation fluid for ^{3}H, and Carbosorb® and Permafluor V® for ^{14}C (Packard Instrument Co.). The efficiency of combustion was at least 90% for ^{3}H and at least 98% for ^{14}C. All samples were counted using a Model 2425 Packard Tri-Carb liquid scintillation spectrometer; the maximum counting error was 1.6%. Counting efficiency was determined with external standardization. Under these conditions, the sensitivity for the detection of residues in tissues was 0.01 ppm for muscle, kidney, and liver, and 0.05 ppm for fat.

Samples of the $^{14}CO_2$-absorbing solution used to trap $^{14}CO_2$ from expired air were counted by mixing 0.5 ml with 1.5 ml of Soluene-350® (Packard Instrument Co.) and 15 ml of the scintillation fluid of ANDERSON and MCCLURE[15].

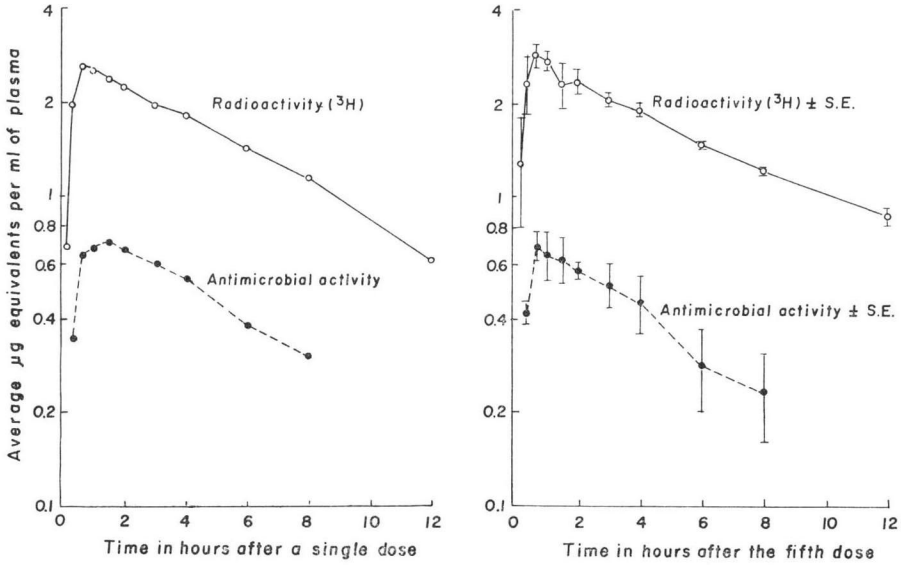
The binding of radioactivity to plasma proteins was determined by a standardized procedure[16].

Results

Dogs

Fig. 2 shows the concentrations of tiamulin-^{3}H equivalents in plasma, and the corresponding antimicrobial activity, after dogs were given either single or multiple 10-mg/kg doses of drug. Maximum concentrations were attained in plasma within 1 hour after dosing and were comparable for both dosage regimens. For the dogs given multiple doses of tiamulin-^{3}H, the average (±S.E.) amounts of radioactivity bound to plasma proteins, as well as the concentrations in plasma, 4 hours after the

Fig. 2. Concentrations of tiamulin-^{3}H equivalents and antimicrobial activity in the plasma of dogs after the administration of a single (10 mg/kg; left) or multiple doses (10 mg/kg per day; right) of drug.



first and fourth doses, were 70±2.7% (1.4±0.13 μg equivalents/ml) and 61±4.8% (1.6±0.19 μg equivalents/ml), respectively. The levels of radioactivity and antimicrobial activity in plasma were roughly parallel for at least 8 hours; the amount of antimicrobial activity was only about one-

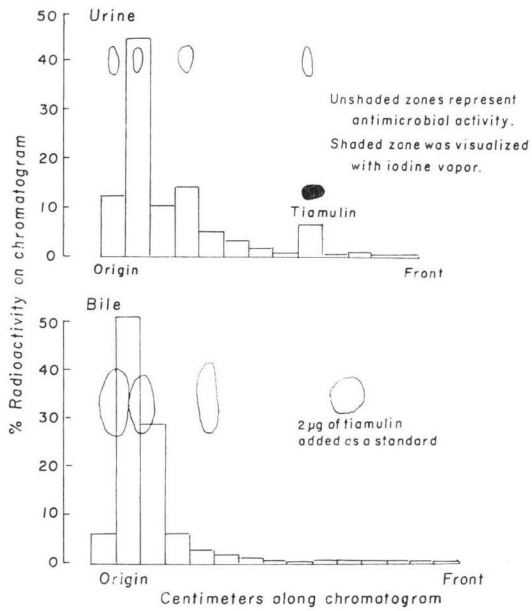
Table 1. Average excretion of radioactivity by dogs after the oral administration of tiamulin-^{3}H (10 mg/kg per day) for 5 consecutive days.

Time of collection	Average % of dose		
	Urine	Feces	Total
Cumulative 24-hour excretions after the first four daily doses	17.80	42.18	59.98
Excretion (0~24 hours) after the fifth dose			
Day 1	4.19	10.63	14.82
Day 2	1.07	9.98	11.05
Day 3	0.22	1.16	1.38
Day 4	0.11	0.57	0.68
Days 5~10	0.22	0.78	1.00
Pan Rinse	0.15	—	0.15
Total±S.E.	23.76 ± 2.21	65.30 ± 1.93	89.06 ± 0.47

Two male and two female dogs were used. The total daily dose was given on the morning of each day in aqueous solution by gavage.

Fig. 3. Chromatograms of samples of 0~1-hour urine (upper) and 2~3-hour bile (lower) collected from an anesthetized dog after the intravenous administration of 2.6 mg/kg of tiamulin-^{3}H.

Thin-layer plates were developed in *n*-butyl acetate - methanol - 28% ammonia (100: 5: 1).



fourth of the total radioactivity, indicating that metabolites of tiamulin were generally less active than the parent compound. The ratio of radioactivity to bioactivity was measured in the urine of dogs each day during the 5-day dosing period. An average ratio of 22.1 ± 1.4 was found, indicating that urinary metabolites of tiamulin-^{3}H were generally less active than the administered drug.

Dogs given multiple 10-mg/kg oral doses of tiamulin-^{3}H excreted radioactivity predominantly in feces (Table 1); similar results were obtained after either single oral (10 mg/kg) or intravenous (3 mg/kg) doses. An average of 85.6% of an oral dose of tiamulin-^{3}H was absorbed by dogs, based on the ratio of the areas under the plasma concentration *versus* time curves after oral and intravenous dosing.

Further evidence that tiamulin was rapidly biotransformed by dogs was obtained in an experiment in which a dog was given a single intravenous dose of tiamulin-^{3}H (2.6 mg/kg); urine and bile were collected and analyzed (Fig. 3). Less than 10% of the radioactivity present in urine during the first hour after dosing was unchanged tiamulin; three other bioactive zones were present at, or near, the origin. The sample of bile collected between 2 and 3 hours after dosing had the greatest concentration of radioactivity. This sample contained essentially no unchanged tiamulin, but three other bioactive zones were again found. Analysis of urine collected from this same dog during the first hour after dosing indicated that 1.8% of the urinary radioactivity was present as tritiated water.

Rats

Rats given single oral 30-mg/kg doses of tiamulin-^{14}C excreted radioactivity in a manner similar to dogs (Table 2). Significantly, 1.3% of the dose was excreted in 48 hours in expired air as $^{14}CO_2$. Analysis of urine collected daily for 11 days from another group of male rats that had been dosed orally with 50 mg/kg of tiamulin-^{3}H indicated that no more than 1.5% of the urinary radioactivity was present as tritiated water.

Table 2. Average excretion of radioactivity by rats after the oral administration of a single dose of tiamulin-^{14}C (30 mg/kg).

Time (Days)	Average % of dose			
	Urine	Feces	$^{14}CO_2$	Total
1	20.73	28.91	1.15	50.79
2	0.74	30.84	0.17	31.75
3	0.24	3.78	—	4.02
4	0.12	0.63	—	0.75
Cage rinse	0.07	—	—	0.07
Total ±S.E.	21.90 ± 4.36	64.16 ± 4.01	1.32 ± 0.11	87.38 ± 2.92

Two male and two female rats were dosed with drug in aqueous solution by gavage.

Four days after they had been dosed, the carcasses contained an average of 2.38 ± 0.23% of the dose (±S.E.).

Pigs

To determine whether a species to be treated therapeutically also excreted $^{14}CO_2$ in expired air, two weanling pigs were given in aqueous solution by gavage single 11-mg/kg doses of tiamulin-^{14}C. During 10 hours, these pigs excreted 1.1 and 0.7% of the dose as $^{14}CO_2$, and the amounts probably would have been greater if the collections had been continued.

Since rats and pigs produced $^{14}CO_2$ in expired air after dosing with tiamulin-^{14}C, some studies were performed in pigs with dual-labeled tiamulin. The average excretion of ^{3}H and ^{14}C in the urine and feces of four pigs that had been given daily 11-mg/kg doses of tiamulin-^{3}H/^{14}C by gavage (in equally divided doses) for 5 consecutive days is shown in Table 3. In 10 days, 93% of the dose was recovered as ^{3}H but only 84% as ^{14}C. Similar results were obtained in other experiments in pigs (95% of the dose recovered in 10 days as ^{3}H and 79% as ^{14}C), in which 10-mg/kg doses of tiamulin-^{3}H/^{14}C were given in the feed in equally divided doses for 10 consecutive days. The smaller recovery of ^{14}C than of ^{3}H is consistent with metabolic cleavage and degradation of the side chain of tiamulin-

Table 3. Average excretion of radioactivity by pigs after the oral administration of tiamulin-$^{3}H/^{14}C$ (11 mg/kg per day) for 5 consecutive days.

Time of collection	Average % of dose					
	^{3}H			^{14}C		
	Urine	Feces	Total	Urine	Feces	Total
Cumulative 24-hour excretions after the first 4 days of dosing.	12.75	32.17	44.92	10.96	30.08	41.04
Excretion (0~24 hours) after the fifth day of dosing						
Day 1	4.25	7.96	12.21	3.48	7.50	10.98
Day 2	1.31	19.48	20.79	1.06	18.34	19.40
Day 3	0.72	6.51	7.23	0.61	5.71	6.32
Day 4	0.37	3.67	4.04	0.33	3.06	3.39
Days 5~10	0.67	2.62	3.29	0.64	2.01	2.65
Pan rinse	0.08	—	0.08	0.07	—	0.07
Total±S.E.	20.15 ± 0.65	72.41 ± 1.77	92.56 ± 2.31	17.15 ± 0.49	66.70 ± 1.83	83.85 ± 2.16

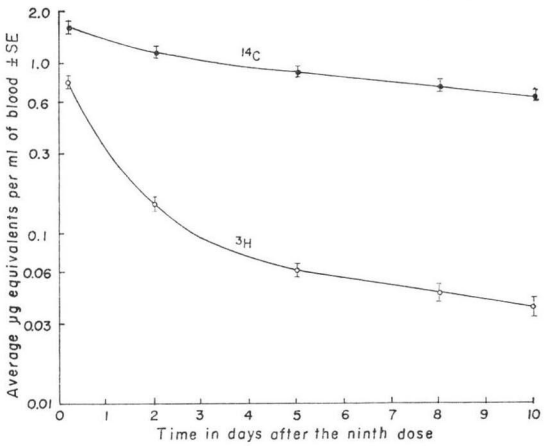
Two male and two female weanling pigs were used. The total daily dose was given in aqueous solution by gavage in equally divided portions (9: 30 a.m. and 3: 30 p.m.).

^{14}C. Analysis of urine, collected from pigs during dosing, and up to 15 days after the last dose of tiamulin-$^{3}H/^{14}C$ (10 mg/kg per day) given in the feed for 10 consecutive days, indicated that no more than 0.3% of the urinary radioactivity was present as tritiated water either during the dosing period or during the subsequent 15 days.

Fig. 4 shows the average concentrations of tiamulin equivalents in the blood of four pigs that had been given by gavage multiple 5.5-mg/kg oral doses of tiamulin-$^{3}H/^{14}C$. After the ninth dose, the concentrations of ^{14}C were consistently much greater than those of ^{3}H, and the initial decline for ^{3}H was more rapid than for ^{14}C.

Fig. 4. Average concentrations of radioactivity (tiamulin equivalents) in the blood of pigs after the oral administration of tiamulin-$^{3}H/^{14}C$ (11 mg/kg per day) for five consecutive days.

Two male and two female pigs were given the drug in aqueous solution by gavage in two equally divided portions (9: 30 a.m. and 3: 30 p.m.).



Residual radioactivity was determined in the blood, plasma, and edible tissues of four pigs, 4 hours and 10 days after the administration of tiamulin-$^{3}H/^{14}C$ by gavage for 5 consecutive days (11 mg/kg per day). The amounts of ^{3}H and ^{14}C were determined and the ratio of ^{14}C to ^{3}H calculated (Table 4). The ratio of ^{14}C to ^{3}H was generally much greater 10 days after the end of dosing than after 4 hours; even after 4 hours, the ratio of ^{14}C to ^{3}H generally was substantially greater than 1.0. Residual radioactivity was also measured 2, 5, 10, and 25 days after the administration of either tiamulin-^{3}H or tiamulin-$^{3}H/^{14}C$ to weanling pigs at a dosage of 10 mg/kg per day in the feed for 10

Table 4. Residual radioactivity (tiamulin equivalents) in the blood, plasma, and tissues of pigs after the oral administration of tiamulin-$^{3}H/^{14}C$ (11 mg/kg per day) for 5 consecutive days.

Sample	4 Hours			10 Days		
	^{3}H (ppm)	^{14}C (ppm)	Ratio of $^{14}C/^{3}H$	^{3}H (ppm)	^{14}C (ppm)	Ratio $^{14}C/^{3}H$
Muscle	0.60±0.06	1.48±0.10	2.58±0.37	0.033±0.004	0.87±0.09	26.5±0.9
Fat	1.18±0.41	2.31±0.42	2.45±0.50	0.083±0.004	1.40±0.07	16.8±0.8
Kidneys	2.74±0.42	4.81±0.34	1.84±0.19	0.056±0.005	0.83±0.05	15.0±0.4
Liver	18.9±0.5	24.3±1.1	1.29±0.02	0.81 ±0.18	2.20±0.29	2.64±0.25
Blood (μg/ml)	0.57±0.08	1.68±0.16	3.06±0.44	0.036±0.005	0.64±0.05	18.0±1.4
Plasma (μg/ml)	0.75±0.12	1.80±0.17	2.52±0.36	0.016±0.002	0.29±0.02	18.2±1.0

Two male and two female weanling pigs were sacrificed at each time.

The total daily dose was given in aqueous solution by gavage in equally divided portions (9: 30 a.m. and 3: 30 p.m.). Each value is the average±S.E.

Table 5. Residual radioactivity (tiamulin equivalents) in the blood, plasma, bile, and tissues of pigs after the oral administration of either tiamulin-^{3}H or tiamulin-$^{3}H/^{14}C$ (10 mg/kg per day) in the feed for 10 consecutive days.

Sample	Average tiamulin equivalents (ppm±S.E.)					
	2 Days	5 Days	10 Days		25 Days	
	^{3}H	^{3}H	^{3}H	^{14}C	^{3}H	^{14}C
Muscle	0.11±0.01	0.05±0.01	0.03±0.00	0.72±0.04	0.01±0.00	0.43±0.03
Fat	0.18±0.02	0.11±0.01	0.07±0.01	0.72±0.13	0.04±0.01	0.91±0.04
Kidneys	0.48±0.07	0.12±0.02	0.06±0.01	0.60±0.05	0.02±0.00	0.23±0.02
Liver	8.77±0.49	2.09±0.30	0.93±0.09	2.19±0.41	0.17±0.03	0.49±0.09
Bile	98.14±22.34	3.30±0.54	0.34±0.09	0.46±0.10	0.05±0.01	0.15±0.00
Blood (μg/ml±S.E.)	0.13±0.03	0.06±0.01	0.03±0.00	0.56±0.03	0.02±0.01	0.32±0.01
Plasma (μg/ml±S.E.)	0.14±0.03	0.04±0.01	0.01±0.00	0.21±0.01	0.00±0.00	0.06±0.00

Four weanling pigs, two of each sex, were sacrificed 2 and 5 days after the end of dosing with tiamulin-^{3}H. Two weanling pigs, one of each sex, were sacrificed 10 and 25 days after the end of dosing with tiamulin-$^{3}H/^{14}C$. The total daily dose was given orally in the feed in equally divided portions (8: 00 a.m. and 3: 30 p.m.).

consecutive days (Table 5). Two days after dosing, concentrations of ^{3}H were greatest in liver and bile. At any of the four times, the concentrations of radioactivity in tissues were greatest in liver. Ten and 25 days after the end of dosing with tiamulin-$^{3}H/^{14}C$, the concentrations of ^{14}C in tissues were consistently greater than the corresponding values for ^{3}H.

Glycogen was isolated and purified from the liver of pigs, 10 and 25 days after the end of a dosage regimen of 10 mg/kg per day of tiamulin-$^{3}H/^{14}C$ in the feed for 10 consecutive days. ^{14}C-Glycogen accounted for 2.1% of the ^{14}C in the liver of one pig at 10 days after the last dose, and 6.5% in one pig after 25 days.

Discussion

The dog and rat, laboratory animal species, and the pig, a species to be treated therapeutically, excreted a radioactive dose of tiamulin similarly in urine and feces. The radioactivity excreted in feces originated in bile, as determined in the present studies in a bile-duct cannulated dog and in other

similar studies in rats (unpublished data). Biliary excretion also appeared to be the predominant excretory route in pigs. In dogs, the metabolic disposition of tiamulin-^{3}H was similar after single and multiple dosage regimens. Tiamulin was rapidly and extensively biotransformed by all animal species studied, generally to metabolites having less bioactivity than tiamulin.

Rats and pigs cleaved the side chain of tiamulin-^{14}C, resulting in the liberation of $^{14}CO_2$. Thus, in dual-labeled studies, a greater percentage of the dose was recovered in the excreta of pigs as ^{3}H than as ^{14}C. In addition, after the last dose, the ratio of ^{14}C to ^{3}H in the tissues and blood of pigs increased with time. Moreover, ^{14}C was found to be present in liver glycogen and urinary urea of pigs (unpublished data). These data indicate that radioactivity from cleavage of the side chain of tiamulin-^{14}C was incorporated into endogenous compounds of the species studied. Since the radioactivity from tiamulin-^{3}H only exchanged with body water to a small extent in pigs, this isotopically-labeled compound is suitable for studying metabolic disposition and tissue residues.

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