Disposition of Oral [^{14}C]Sulfathiazole in Swine

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Disposition of oral sulfathiazole was studied in swine. Pigs were slaughtered 6, 12, 24, and 48 h after an oral dose of [^{14}C]sulfathiazole (two at each time period). Excretion of ^{14}C was rapid (>90% in 48 h), primarily via the urine. Metabolites isolated and characterized by ^{1}H NMR and FAB MS were N^4-acetylsulfathiazole from urine, kidney, liver, blood, and muscle; N^4-glucoside of sulfathiazole from muscle; and an apparent diconjugate from liver, a glucuronide of N^4-acetylsulfathiazole. Quantitation was accomplished by HPLC analysis of samples (extracts of tissue and urine) spiked with the reference compounds. Peaks corresponding to the retention time of the reference compounds were trapped and assayed for ^{14}C. Sulfathiazole and N^4-acetylsulfathiazole were the principal ^{14}C-labeled compounds in urine and kidney. If present, the glucoside or glucuronide represented <5% of the ^{14}C in urine. Liver and muscle contained significant amounts of sulfathiazole, N^4-acetylsulfathiazole, and N^4-glucoside of sulfathiazole. Quantitation of the diconjugate was not attempted. The amounts isolated suggest it was a minor metabolite; however, instability during isolation was apparent. On the basis of the results, liver, kidney, and urine are potential target tissues for a residue monitoring program and the nature of the metabolite present in the tissue must be considered when an assay procedure is selected.

Keywords: *Sulfathiazole; swine; metabolism*

INTRODUCTION

Sulfathiazole is currently approved as a feed additive in swine rations (*1995 Feed Additive Compendium*, Miller Publishing Co., Minnetonka, MN). Pharmacokinetic studies in swine have shown that sulfathiazole is rapidly absorbed from the gastrointestinal tract and rapidly excreted via the urine, primarily as unchanged sulfathiazole and its N^4-acetyl derivative (Bourne et al., 1977; Duddy et al., 1984; Koritz et al., 1977). Righter et al. (1971), using the Tishler method for determination of free sulfonamides (Tishler et al., 1968), reported tissue residue depletion following a therapeutic oral dose (330 mg/kg of body weight) of sulfathiazole to swine. Muscle, liver, kidney, and fat were all below 0.1 ppm (level of detection) by 10 days after withdrawal. None of the published reports of sulfathiazole disposition in swine have used radiolabeled sulfathiazole. The objectives of the study reported here were to determine the distribution of ^{14}C at various times after an oral dose of [^{14}C]sulfathiazole to swine and to characterize and quantitate the ^{14}C-labeled products in tissues and urine.

MATERIALS AND METHODS

Animals and Animal Handling. Castrated male swine of mixed breeding weighing 15–20 kg were obtained from the North Dakota State University swine herd. Immediately after the animals were transported to this laboratory, they were identified by tattooing and treated for parasites with ivermectin as per label directions. Their diet was a conventional corn–soybean oil meal growing ration. Over a 10–14 day period the animals were gradually acclimated to being held in a metabolism cage and trained to consume their ration as "dough balls". Dough balls were formed by adding wheat flour to the ration (1:3) and sufficient water for the mixture to be formed into 3–4 cm spheres.

Dosing Material and Dosing Procedure. Sulfathiazole (4-amino-N-2–thiazoylbenzenesulfonamide) uniformly labeled with ^{14}C in the aniline ring ([^{14}C]sulfathiazole) was obtained from Sigma Chemical Co., St. Louis, MO. Specific activity as received was 8.4796 × 10^7 dpm/mg with an indicated radiopurity of >98% (HPLC analyses). The desired specific activity for the dosing material was obtained by mixing the appropriate amount of unlabeled sulfathiazole (Sigma) with the original [^{14}C]sulfathiazole and recrystallizing from methanol. Specific activities of the dosing materials were determined by accurately weighing approximately 200 mg of the recrystallized [^{14}C]sulfathiazole, dissolving in 100 mL of methanol, and assaying by liquid scintillation. A single UV-absorbing peak was observed upon HPLC analyses of the recrystallized [^{14}C]-sulfathiazole, and the peak area contained >96% of the ^{14}C recovered from the HPLC run.

The actual dose was prepared by mixing the appropriate amount (by weight) of the recrystallized [^{14}C]sulfathiazole with approximately 200 g of the feed–flour mixture. The dose was formed into dough balls as previously described. The mixing pan was then "rinsed" with an additional 100 g of feed mix, which was also formed into dough balls. The total dose was consumed in approximately 20 min. Unconsumed [^{14}C]sulfathiazole was determined by assaying a rinse of the cage front and all utensils used for mixing. This was always an insignificant amount (<0.01% of the dose). When the pigs were fed after dosing, the diet was the corn–soybean oil meal ration. Table 1 outlines the regimen for the dosings.

Sample Collection and Handling. (Animal handling and care complied with USDA, ARS Guidelines for Care of Research Animals and protocols approved by the local Animal Care Committee.) Pigs were held in elevated metabolism stalls (Pekas, 1968) from shortly before dosing until slaughter. Urine was collected quantitatively on ice using attached urinals (Paulson and Cottrell, 1984). Pigs were anesthetized with halothane and washed before killing by exsanguination (heart puncture after injection of 30 000 units of heparin).

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Table 1. Regimen for Oral Dosing of Swine with [^{14}C]Sulfathiazole

animal ID	date of dosing	body wt (kg)	sp act. of dose[a] (dpm/μg)	from dosing to slaughter (h)
241	Dec 13, 1989	32	1040	6
247	May 15, 1990	39	973	6
239	Nov 7, 1990	38	1040	12
246	March 27, 1990	36	1040	12
235	Sept 26, 1989	32	1012	24
243	Jan 23, 1990	34	1040	24
242	Dec 19, 1990	35	1040	48
250	May 22, 1990	37	973	48
236	Sept 29, 1989	28	control	b
244	Jan 30, 1990	31	control	b

[a] The mass of the dose ranged from 99.9 to 101.3 mg/kg of body weight. [b] Before slaughter, swine 236 and 244 were held in a metabolism crate for 16 and 18 h, respectively.

Samples of the following tissues were collected after slaughter: blood from heart puncture, urine from bladder, bile, gall bladder, heart, lungs, liver, kidney, spleen, skeletal muscle (loin), dermal fat, visceral fat, and the gastrointestinal tract with contents. The entire eviscerated carcass was ground (Autio GP-801B grinder with a 1 cm die, Autio Co., Astoria, OR) and mixed thoroughly (Hobart L-800 mixer, Hobart Manufacturing Co., Troy, OH) before sampling. Tissue samples were held on ice until they were either homogenized in a Waring type blender or ground with a conventional meat grinder before storage at −28 °C for later analyses.

Radioassays. Assays of tissues and blood were accomplished with a Model 307 sample oxidizer (Packard Instruments, Downers Grove, IL). Approximately 0.5 g of ground tissue or blood was accurately weighed into a small cellulose cup. An additional absorbent pad was placed in the cup when blood was analyzed (Combusto-Cone and Combusto-Pad, Packard Instruments). Weighed samples were dried overnight at 37 °C before combusting. Fat was assayed by placing approximately 0.5 g in a scintillation vial, adding 9 mL of Carbo-Sorb, allowing to stand overnight, shaking vigorously, adding 12 mL of Permafluor, and counting by liquid scintillation (Packard Instruments). Samples were done in triplicate (except six samples of each carcass were combusted). When the total variation of the assay of a sample was >10% of the mean, three additional samples were assayed and the mean of all six was used. For fat, carcass, and gastrointestinal tract the allowable variation was 20%. When disintegrations per minute per gram of tissue was <100, variation up to 30% was allowed before reassays were done.

Synthesis of Reference Compounds. The N^4-acetylsulfathiazole (4-acetamido-N-2-thiazolylbenzenesulfonamide) and the N^4-glucuronic acid conjugate of sulfathiazole were synthesized as described by Nelson et al. (1987). Spectral properties of N^4-acetylsulfathiazole were as follows: negative FAB-MS M − 1 m/z 296, M − 99 m/z 198; ^{1}H NMR (400 MHz, CD_3OD) δ 2.13 (s), 6.71 (d, J = 4.6 Hz), 7.09 (d, J = 4.6 Hz), 7.69 (d, J = 8.5 Hz), 7.81 (d, J = 8.5 Hz). Spectral properties of sulfathiazole−N^4-glucuronide were as follows: negative FAB-MS, M − 1 m/z 430, (M − 1) + (Na − 1) m/z 452, M − 99 m/z 332; ^{1}H NMR (400 MHz, CD_3OD) δ 4.61 (J = 8.6 Hz, anomeric, other glucuronide protons were not adequately resolved for assignments), 6.65 (d, J = 4.6 Hz), 6.81 (d, J = 8.0 Hz), 7.04 (d, J = 4.6 Hz), 7.64 (d, J = 8.0 Hz). The N^4-glucoside of sulfathiazole was synthesized using the method for the N^4-lactose conjugates of sulfonamides (Paulson et al., 1992). Instead of using HPLC for cleanup, the product was forced out of solution with the addition of ether. The oil was washed with ether and acetone. Ethyl acetate was added, and the mixture was cooled to −10 °C. A white solid formed after several days that had a mp of 115−122 °C. Spectral properties were as follows: positive FAB-MS, M + 1 m/z 418, negative FAB-MS, M − 1 m/z 416, M − 99 m/z 318; ^{1}H NMR (400 MHz, CD_3OD) δ 4.59 (d, J = 8.6 Hz, anomeric, other glucoside protons were not adequately resolved for assignments), 6.66 (d, J = 4.6 Hz), 6.81 (d, J = 8.8 Hz), 7.05 (d, J = 4.6 Hz), 7.65 (d, J = 8.8 Hz).

Isolation and Characterization of Metabolites. For characterization of ^{14}C-labeled compounds, tissues were extracted with methanol containing 10% v/v of a 1 mM, pH 7.2, phosphate buffer. The methanol was removed with a rotary evaporator, and the aqueous remainder was back-extracted with hexane. Radioactive fractions were then isolated using various combinations of low-pressure chromatography and HPLC. For low-pressure columns, 100−200 mesh Porapak Q (Waters Division of Millipore, Milford, MA) was used in 1.6 × 25 cm columns. A typical elution sequence was pH 7.0 PO_4 buffer for 15 min, a linear gradient to 100% methanol in 60 min, and 100% methanol for 15 min (flow rate 2.5 mL/min). For HPLC, a C_{18} Nova-Pak (8 × 100 mm, 4 μm mesh; Waters) was used. A typical elution sequence was pH 7.0 PO_4 buffer for 5 min, linear gradient to 30% methanol in 27 min, 30% methanol for 5 min, linear gradient to 100% methanol in 5 min, and 100% methanol for 8 min (flow rate 2.5 mL/min). Elution from these columns was monitored with a radioactive flow detector. Characterization of the radioactive fractions was by mass spectrometry and ^{1}H NMR (VG Autospec, Bruker AM 400). In the final chromatography step before spectroscopy, the elution system was a water/methanol gradient.

Urine samples were applied directly to a Porapak Q column which was then eluted with a pH 7.0 phosphate buffer/methanol gradient. The radioactive fraction from this column was subjected to HPLC and Porapak Q as with the tissue extracts.

For quantitation of metabolites in tissues a slight modification of the matrix solid phase dispersion method (MSPD) of Long et al. (1990) was used to extract tissues. Wet tissues were ground with C_{18} HPLC packing material (ratio 1:4) in a mortar. The mass of tissue varied with the concentration of ^{14}C so that a minimum of 40 μg of sulfathiazole equivalents was present (up to 10 g of tissue). In the procedure described by Long et al. (1990), the C_{18} tissue mixture was placed in a chromatographic column and washed with hexane followed by methylene chloride. They reported over 80% recovery in the methylene chloride when tissues spiked with sulfathiazole were extracted according to this procedure. However, with muscle tissue from an animal in our study, less than 40% of the ^{14}C could be eluted with methylene chloride. Elution with methanol was necessary for satisfactory recoveries of ^{14}C. Therefore, our procedure was to elute with hexane (10 mL for a 1 g sample; 50 mL for a 10 g sample) followed by methanol (100 mL for a 1 g sample and 300 mL for a 10 g sample of tissue). Initial concentration of the methanol eluant was done with a rotary vacuum evaporator with the final concentration to less than 1 mL with either a Speed Vac (Savant Instruments, Farmingdale, NY) or a stream of nitrogen. Precipitate which formed in some extracts upon concentration was removed by centrifugation.

The concentrated eluants were then spiked with approximately 30 μg each of sulfathiazole, N^4-glucuronide of sulfathiazole, N^4-acetylsulfathiazole, and N^4-glucoside of sulfathiazole. The spiked eluants were chromatographed on a C_{18} Nova-Pak column. Solvent A was water with 1% acetic acid, and solvent B was methanol with 1% acetic acid. Flow rate was 2 mL/min with a program of 5 min of solvent A, linear gradient to 15% B in 15 min, and 8 min at 15% B. The eluate was monitored by UV at 254 nm. Peaks corresponding to the retention times of the four compounds added to the extract were trapped and assayed for ^{14}C. The remainder of the eluate was also collected and assayed. Values from these assays were used to compute total recovery from the column and the percentage of each metabolite. For muscle and liver extracts, the injection volume was 200−300 μL (10 000−20 000 dpm [^{14}C]sulfathiazole equivalents) and for kidney it was 20−40 μL (approximately 40 000 dpm [^{14}C]sulfathiazole equivalents).

Urine samples were analyzed on the same HPLC system. Immediately before injection, the urine was adjusted to pH 6 with HCl. For injections, acidified urine (20−50 μL) was

Table 2. Distribution (Percentage of Dose)[a] of ^{14}C at Time of Sacrifice following Oral Dosing of Swine with [^{14}C]Sulfathiazole

item	time from dosing until sacrifice			
	6 h	12 h	24 h	48 h
excreted in urine	38.7 ± 18.3	55.8 ± 6.8	73.6 ± 14.9	87.8 ± 5.4
bladder urine	5.06 ± 5.30	2.86 ± 1.47	0.90 ± 0.96	0.03 ± 0.02
feces	0.06 ± 0.04	0.17 ± 0.24	2.8 ± 3.7	3.4 ± 2.7
GI tract and contents	32.5 ± 17.7	13.2 ± 6.5	15.6 ± 15.6	2.7 ± 1.8
carcass	12.9 ± 1.58	5.4 ± 2.0	1.7 ± 0.43	0.16 ± 0.05
cage and carcass wash	0.8 ± 0.1	2.2 ± 2.8	0.6 ± 0.05	0.1 ± 0.03
total recovery	93.8 ± 8.4	82.4 ± 2.6	93.8 ± 1.4	94.5 ± 6.2

[a] $n = 2$ for all values.

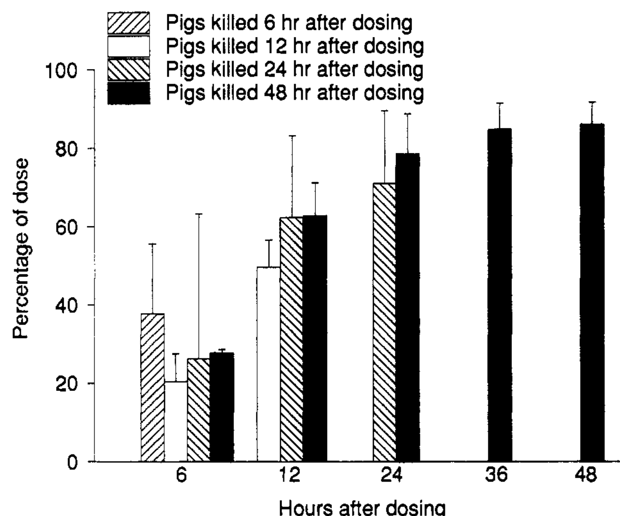

Figure 1. Cumulative urinary excretion of ^{14}C following oral dosing of swine with [^{14}C]sulfathiazole.

drawn into a syringe that contained approximately 10 μg each of unlabeled sulfathiazole, N^4-glucuronide of sulfathiazole, N^4-acetylsulfathiazole, and N^4-glucoside of sulfathiazole (total injection volume 40–90 μL). With urines collected more than 24 h after dosing, the samples were concentrated on C_{18} Sep-Pak (Waters) before chromatography. Duplicate quantitative HPLC runs were made with each urine or tissue extract sample.

RESULTS AND DISCUSSION

Table 2 shows the distribution of ^{14}C at time of sacrifice. Total recoveries of ^{14}C averaged 90.1% (range 80.6–99.7%). As expected, urine was the principal route of excretion. Urinary excretion was rapid, as shown in Figure 1. Table 3 shows ^{14}C concentrations (expressed as parts per million of sulfathiazole equivalents) in various tissues at time of sacrifice. Although ^{14}C concentrations decreased rapidly with time, they were still greater than 1 ppm in kidney and liver at 48 h after dosing.

Table 3. Concentrations (Parts per Million)[a] of ^{14}C in Tissues at Time of Sacrifice following Oral Dosing of Swine with Sulfathiazole

tissue	time from dosing until sacrifice			
	6 h	12 h	24 h	48 h
bladder urine	8055 ± 3343	2599 ± 759	434 ± 99	31.8 ± 2.02
kidneys	177 ± 49.7	89.0 ± 24.9	20.4 ± 5.3	1.87 ± 0.41
liver	34.7 ± 7.4	15.8 ± 3.8	4.94 ± 2.42	1.05 ± 0.07
bile	43.8 ± 19.8	36.6 ± 19.7	5.22 ± 2.04	0.74 ± 0.33
blood	34.4 ± 7.0	15.6 ± 5.1	3.92 ± 2.18	0.30 ± 0.11
heart	25.4 ± 3.6	10.4 ± 3.0	2.74 ± 1.44	0.22 ± 0.11
lungs	24.4 ± 3.1	10.7 ± 3.5	2.78 ± 1.58	0.26 ± 0.12
skeletal muscle	13.3 ± 1.7	5.39 ± 1.8	1.56 ± 1.0	0.095 ± 0.035
visceral fat	11.7 ± 0.7	11.0 ± 9.0	1.83 ± 1.5	0.14 ± 0.03
dermal fat	6.78 ± 0.29	3.22 ± 1.38	0.98 ± 0.58	0.060 ± 0.014

[a] Expressed as parts per million of sulfathiazole equivalents. $n = 2$.

^{14}C-containing compounds were characterized (^1H NMR and FAB mass spectrometry) in samples from the swine killed 6 h after dosing. Sulfathiazole and the N^4-acetylsulfathiazole were isolated and characterized from urine, kidney, liver, blood, and muscle. The N^4-glucoside of sulfathiazole was isolated and characterized from muscle tissue.

An apparent diconjugate characterized as a glucuronic acid conjugate of N^4-acetylsulfathiazole was isolated from the liver. Negative FAB-MS of the sample exhibited M − 1 m/z 472 and (M − 1) + (Na − 1) m/z 494. NMR evidence for the N^4-acetyl derivative included a singlet at δ 2.15 (the standard N^4-acetylsulfathiazole absorbs at δ 2.13), a doublet at δ 7.73 (J = 8.5 Hz), a doublet at δ 7.80 (J = 8.5 Hz) (N^4-acetylsulfathiazole absorbs at 7.69 and 7.81, respectively), and doublets at δ 7.59 and 7.66 (J = 3.6 Hz) characteristic of the thiazole ring (N^4-acetyl at 6.71 and 7.09, J = 4.6 Hz). Evidence for a glucuronic acid conjugate included a doublet at δ 5.40 (J = 9.6 Hz). The glucuronide may be attached to the N' or N-hetero nitrogen. We were unable to conclusively assign the site of glucuronide conjugation from NMR data. The amount of this compound isolated suggests it was present in very low concentration; however, changes in the chromatographic behavior of this fraction upon rechromatography suggest instability during the isolation process.

Table 4 shows the profile of [^{14}C]labeled compounds in urine at various intervals after dosing. Values in the table are expressed as a percentage of the total ^{14}C recovered from the eluate of the HPLC column. Average

Table 4. ^{14}C Activity[a] in Urine of Swine Dosed Orally with [^{14}C]Sulfathiazole That Cochromatographed with Reference Compounds

metabolite	time after dosing				
	0–6 h (n = 7)	6–12 h (n = 6)	12–24 h (n = 4)	24–36 h (n = 2)	36–48 h (n = 2)
very polar[b]	1.2 ± 1	2.4 ± 2.8	1.6 ± 0.2	4.3 ± 3.1	0.6 ± 0.5
N^4-glucoside of sulfathiazole	1.5 ± 0.8	3.1 ± 3.3	1.2 ± 0.3	3.2 ± 2.8	3.4 ± 0.8
sulfathiazole	59.7 ± 6.0	58.8 ± 7.6	37.7 ± 1.4	39.1 ± 8.6	24.5 ± 2.1
N^4-acetylsulfathiazole	32.8 ± 4.2	31.0 ± 11.6	53.1 ± 3.2	42.3 ± 10.9	60.6 ± 0.8
eluted between peaks	4.7 ± 2.9	4.7 ± 2.2	6.4 ± 2.1	11.1 ± 1.1	10.8 ± 2.6

[a] Synthesized reference compounds were added to the urines prior to HPLC analysis. Peaks with retention times of the reference compounds were trapped and assayed for ^{14}C. Values are percentages of the total ^{14}C recovered from the HPLC column. [b] Components of this fraction had very little retention and included the N^4-glucuronide of sulfathiazole; however, other UV-absorbing compounds were also present.

Table 5. ^{14}C Activity[a] in Extracts of Tissue from Swine Dosed Orally with [^{14}C]Sulfathiazole That Cochromatographed with Reference Compounds

	time from dosing until sacrifice					
	6 h			12 h		
metabolite	kidney	liver	muscle	kidney	liver	muscle
very polar[b]	9.8 ± 1.8	6.9 ± 1.2	0.8 ± 0	7.6 ± 0.64	6.9 ± 3.5	2.2 ± 0.21
N^4-glucoside of sulfathiazole	2.6 ± 0.5	38.6 ± 2.5	56.9 ± 9.1	4.0 ± 0.64	37.0 ± 4.6	58.2 ± 5.2
sulfathiazole	34.0 ± 2.2	18.6 ± 0.35	17.1 ± 2.5	31.2 ± 2.4	14.2 ± 1.8	16.3 ± 7.1
N^4-acetylsulfathiazole	37.8 ± 1.1	20.6 ± 1.6	19.4 ± 6.2	43.8 ± 6.2	24.8 ± 7.8	13.1 ± 3.8
eluted between peaks	15.8 ± 1.0	15.2 ± 2.6	5.85 ± 0.35	13.6 ± 5.0	17.6 ± 1.6	10.2 ± 2.1

[a] Reference compounds were added to extracts prior to HPLC analysis. Peaks with retention times of the reference compounds were trapped and assayed for ^{14}C. Values are percentages of the total ^{14}C recovered from the HPLC column. $n = 2$. [b] Components of this fraction had very little retention and included the N^4-glucuronide of sulfathiazole; however, other UV-absorbing compounds were also present.

recovery from the HPLC column expressed as a percentage of the calculated amount injected was 103.2 ± 9.2%. On the basis of cochromatography, sulfathiazole and N^4-acetylsulfathiazole were the principal ^{14}C-labeled compounds in urine. If present, the N^4-glucoside or glucuronide was <5%. With time after dosing, the ratio of acetylsulfathiazole to sulfathiazole increased from approximately 0.5 to 2.5.

Table 5 shows the profile of ^{14}C-labeled compounds in kidney, liver, and muscle from animals killed 6 or 12 h after dosing. Recoveries of ^{14}C from tissues by the extraction procedure were as follows: kidney, 91.4 ± 3.4%; muscle, 85.3 ± 4.3%; and liver, 83.9 ± 2.3%. These calculations are based on combustion analyses of the residue remaining after extraction. Sulfathiazole and N^4-acetylsulfathiazole were the principal ^{14}C compounds in kidney tissues, whereas in liver and muscle, the N^4-glucoside of sulfathiazole was also present in significant quantities (on the basis of cochromatography).

These results suggest that kidney, liver, and bladder urine are potential target tissues for monitoring for sulfathiazole residue in swine offered for slaughter. Obviously, any monitoring assay would have to take into consideration that the sulfathiazole residues may be present as some type of conjugate.

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