Fate of [^{14}C]Arsanilic Acid in Pigs and Chickens

Peter W. Aschbacher* and Vernon J. Feil

Biosciences Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, North Dakota 58105

Arsanilic acid uniformly labeled with ^{14}C in the benzene ring was used to determine the metabolic fate of oral arsanilic acid in pigs and chickens. Arsanilic acid was well absorbed in both species, and urine was the predominant route of excretion. The bile was a minor (<5% of the dose) route of excretion in pigs; however, biliary excretion was not measured in roosters. Arsanilic acid, *N*-acetylarsanilic acid, and (4-acetamidophenyl)dimethylarsine oxide were isolated from pig urine (17–39%, 15–29%, and <5% of urinary ^{14}C, respectively). Only 25% of the ^{14}C in pig feces was extractable, and no metabolites could be isolated. Arsanilic acid was the only radioactive compound isolated from urine of colostomized roosters, and there was no suggestion of other metabolites from the isolation scheme employed. No attempt was made to isolate ^{14}C compounds in feces from colostomized roosters or in excreta from normal roosters.

INTRODUCTION

Arsanilic acid (Figure 1) is approved for use in feed for swine and poultry at concentrations up to 0.01% of the ration. Some information concerning rates of excretion of arsanilic acid from pigs is available; however, information about biotransformations is inconclusive (Overby and Frost, 1960). Chickens excrete arsanilic acid rapidly with no biotransformation (Frost, 1953; Frost et al., 1955; Moody and Williams, 1964; Overby and Straube, 1965). Data about absorption from the gastrointestinal tract of chickens are not available. The objectives of the study reported here were to determine rates and routes of excretion of ^{14}C after oral administration of [^{14}C]arsanilic acid to pigs and chickens and to characterize the ^{14}C-labeled compounds in the excretory products.

MATERIALS AND METHODS

Chemicals. Arsanilic acid uniformly labeled with ^{14}C in the benzene ring was obtained from New England Nuclear (2.67 mCi/mmol; radiopurity 97% as received). Unlabeled arsanilic acid was purchased from Eastman Kodak Co. The unlabeled arsanilic acid was recrystallized once (20 mg/mL in 80 °C water; crystals formed overnight in a refrigerator). The dosing material was prepared by mixing the recrystallized arsanilic acid with ^{14}C-labeled arsanilic acid to achieve the desired activity (approximately 2×10^6 dpm/mg for pig experiments and 4×10^5 dpm/mg for rooster experiments). The dosing material was then recrystallized three times from water/methanol (1:1, 80 °C). Specific activities were determined after each crystallization by drying crystals in a vacuum oven to a constant weight. Triplicate samples of crystals (3–10 mg) were weighed and dissolved, and the solution was assayed for radioactivity. The specific activities (dpm $\times 10^6$/mg) after each crystallization were 1.973 ± 0.093, 2.09 ± 0.023, and 2.175 ± 0.117 for the dosing material used for pigs and 0.4077 ± 0.0112, 0.4131 ± 0.0044, and 0.4291 ± 0.006 for the dosing material used for roosters. The relatively constant specific activities were used as evidence of radiopurity.

Triethylammonium acetate (0.1% and 0.5% for HPLC mobile phases) was made by adding glacial acetic acid to aqueous triethylamine solutions (0.1% and 0.5% v/v) until the desired pH was attained.

Dimethylphenylarsine was purchased from Organometallics, Inc., and was purified by distillation: mass spectrum, *m/z* (rel intensity) 182 (36), 167 (52), 91 (100); ^{1}H NMR (400 MHz) δ 1.15 (6 H, s, CH_3), 7.25–7.31 (3 H, m, 3,4,5-aryl), 7.46 (2 H, dd, *J* = 1.4, 8.0 Hz, 2,6-aryl). Oxidation with an excess of 30% hydrogen peroxide yielded dimethylphenylarsine oxide: mass spectrum, *m/z* (rel intensity) 198 (5), 183 (7), 91 (100); ^{1}H NMR (400 MHz)

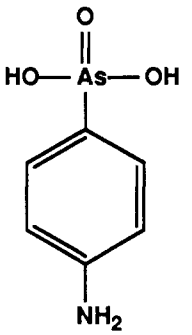

Figure 1. Structure of arsanilic acid [(4-aminophenyl)arsonic acid].

δ 1.97 (6 H, s, CH_3), 7.61–7.64 (3 H, m, 3,4,5-aryl), 7.81 (2 H, dd, *J* = 1.5, 7.9 Hz, 2,6-aryl).

Animals and Animal Treatment. Pigs (barrows) of mixed breeding were obtained from a swine herd at North Dakota State University. Their diet was conventional corn (80.6%) and soybean meal (16.5%) mix with added mineral and vitamins (2.9%). The pigs were acclimated to metabolism stalls (Pekas, 1968) for at least 3 weeks before they were dosed with [^{14}C]-arsanilic acid. A urinal (Paulson and Cottrell, 1984) was used to ensure complete separation of urine and feces during sample collection. Oral dosing was accomplished by pipetting an aqueous solution of [^{14}C]arsanilic acid onto a portion of a morning ration. The feed had been moistened until it could be formed into small balls (20% wheat flour by weight had been added to the feed to facilitate formation of the feed balls). Pigs had been trained to consume their ration rapidly in this form. Table I outlines the pig experiments. The mass of the radioactive dose was approximately equal to the intake from 0.01% arsanilic acid in half of a normal daily ration. Two of the pigs (226 and 230) had been receiving 0.01% unlabeled arsanilic acid in their diet previous to and after the radioactive dose. This was done to increase the mass of metabolites present to facilitate isolation and identification of metabolites.

For the bile collection experiment, a pig was fitted with a biliary cannula and a duodenal reentry cannula. The pig was allowed to recover from the surgery for 5 days before being dosed with [^{14}C]arsanilic acid. Previously collected bile was infused into the intestines during recovery and collection period.

Six white Leghorn roosters (1.2–1.5 kg) were used for the experiments with chickens. The diet was a commercial 20% protein mash designed for laying hens. Separate urine–feces collections were accomplished in three roosters by colostomies (Paulson, 1969). Feces or excreta (urine plus feces) were collected on stainless steel pans under the wire mesh floor of the cages. For

Table I. Outline of [^{14}C]Arsanilic Acid Experiments with Pigs

	pig identification			
	118	225	226	230
pig wt, kg	70	34	35	26
[^{14}C]arsanilic acid dose				
mass, mg	217	84.2	84.2	50
radioactivity, dpm	3.82×10^8	1.83×10^8	1.83×10^8	1.12×10^8
unlabeled arsanilic acid in ration,[a] %	0.00	0.00	0.01	0.01
bile duct cannulated	no	no	no	yes
hours from dosing until sacrifice	96	96	96	76

[a] Pigs 226 and 230 were fed unlabeled arsanilic acid for at least 2 weeks before dosing with [^{14}C]arsanilic acid. Feeding of unlabeled arsanilic acid continued through the collection period.

dosing with [^{14}C]arsanilic acid, gelatin capsules were filled with chicken feed and the arsanilic acid solution was pipetted onto the feed. Each bird received two capsules containing a total of 11.45 mg of arsanilic acid containing 4.6672×10^6 dpm of ^{14}C. The mass of the [^{14}C]arsanilic acid dose was approximately 1.5 times the expected daily intake from free choice feed containing 0.01% arsanilic acid, and this was the only arsanilic acid the roosters received. The birds were sacrificed 72 h after receiving the radioactive dose, and selected tissue samples were collected.

^{14}C Assays and Detection. All quantitation of ^{14}C was done by liquid scintillation techniques. Urine and bile (0.5 mL) were assayed directly in aqueous type scintillation cocktail. Feces or excreta were slurried, freeze-dried, and assayed by oxidation and trapping of the resulting $^{14}CO_2$ (Packard Instrument Co. Model 306 sample oxidizer). Tissue samples were ground, freeze-dried, and assayed by oxidation. The entire eviscerated carcasses were also ground and mixed, and a sample was freeze-dried for ^{14}C assay. Oxidations of carcass samples were done in quadruplicate; other samples were done in triplicate. Radioactivity in chromatography eluants was detected by liquid flow monitors (Packard Instrument Model 320E or 7500), but quantitation was by liquid scintillation techniques.

Chromatography. Low-pressure liquid chromatography was accomplished with a 2.4 × 25 cm column of Porapak Q (100–200 mesh, Waters Division of Millipore). After the sample was applied, the column was eluted with water followed by either methanol or a linear gradient from 1% acetic acid in water to methanol. In the latter case the sample was applied as an aqueous solution at pH 4.5. HPLC systems were as follows: C-18 Radial Pak (8 × 100 mm, 4 μm Novapak, Waters Division of Millipore) with a mobile phase of 1% acetic acid in water or a linear gradient of the same to 100% methanol; C-18 analytical column (4.6 × 250 mm, 5 μm Alltech Associates) with a linear gradient from 1% acetic acid in water to 100% methanol; Cyclobond 1 (4.6 × 250 mm, Advanced Separation Technologies) with a mobile phase of 0.5% triethylammonium acetate (TEAA), pH 4.5/methanol, 2:8 (Cyclobond A) 3:7 (Cyclobond B), or 0.1% TEAA, pH 4.5/methanol, 7:3 (cyclobond C). These chromatography systems were used in sequence as shown in Figure 2 to isolate urinary metabolites.

Spectral Data. Mass spectra (MS) were obtained with a Varian MAT CH-5 spectrometer interfaced with a SS-200 data system and equipped with an AMD Intectra combination source and post accelerator. An Ion Tech saddle field gun operated at 8 keV with xenon provided the beam of fast atoms to bombard glycerol solutions of the compounds for negative fast atom bombardment (-FAB) mass spectrometry. Usually 1,8-bis(dimethylamino)naphthalene (Proton Sponge, Aldrich Chemical Co. Inc.) was added to the glycerol solution. Electron impact spectra were obtained at 70 eV by using a direct insertion probe.

NMR spectra were taken with a Brucker AM-400 instrument in deuteromethanol referenced to internal trimethylsilane.

RESULTS AND DISCUSSION

Pigs. The distribution of ^{14}C after an oral dose of [^{14}C]-arsanilic acid is shown in Table II. Figure 3 shows

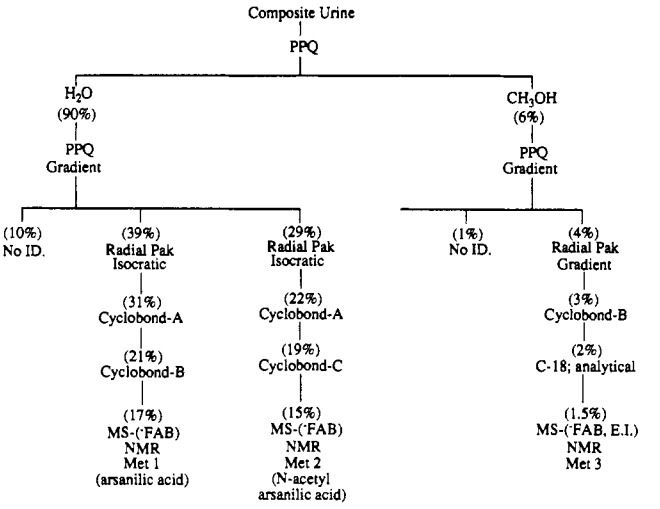

Figure 2. Procedures used to isolate and characterize urinary metabolites from pigs given [^{14}C]arsanilic acid. Numbers in parentheses are recoveries of ^{14}C in that fraction expressed as a percentage of the ^{14}C in the urine.

Table II. Distribution of ^{14}C after Oral Dosing of Pigs with [^{14}C]Arsanilic Acid[a]

	% of dose			
	pig 118	pig 225	pig 226	pig 230
urine	65.8	47.7	63.2	58.9
feces	24.6	42.2	25.8	18.2
bile				4.7
GI tract	2.7	1.2	1.4	10.5
carcass	1.7	0.8	1.0	3.0
liver	0.9	1.2	0.9	1.9
total recovery	95.7	93.1	92.3	97.3

[a] All pigs received a single dose of [^{14}C]arsanilic acid; however, before receiving their radioactive dose and during the collection period, pigs 226 and 230 received feed containing 0.01% arsanilic acid. The time from dosing with [^{14}C]arsanilic acid to sacrifice was 96 h for pigs 118, 225, and 226 and 76 h for pig 230.

cumulative excretion of ^{14}C. Excretion via the urine was very rapid with 35–40% of the dose excreted within 12 h of dosing. By 48 h after dosing, total urinary excretion essentially reached a plateau. Fecal excretion was not complete by 96 h after dosing as indicated by the 2–3% of the dose remaining in the gastrointestinal tract. Bile was a relatively minor route of excretion. Consumption of unlabeled arsanilic acid before and after the dose of [^{14}C]arsanilic acid (pigs 226 and 230) did not have a detectable influence on excretion of ^{14}C during the time period observed.

The concentration of ^{14}C in various tissues at sacrifice is shown in Table III. For a convenient reference, the values are expressed as equivalents of arsanilic acid (which assumes all ^{14}C remains as arsanilic acid); however, it should be emphasized these are measurements of ^{14}C and not arsanilic acid. In addition, these are ^{14}C residues from a single dose of [^{14}C]arsanilic acid and do not reflect residues that might occur in an animal that had received feed containing 0.01% arsanilic acid for several days before withdrawal of the arsanilic acid. In all animals, the liver contained the highest concentration of radioactivity.

Three radioactive compounds were isolated from urine and characterized. Figure 2 shows the results of the various chromatographic procedures that were used. Recoveries in each chromatographic peak (as detected by a radioactivity monitor) are shown in the figure. These values are minimum estimates of the quantity of each metabolite

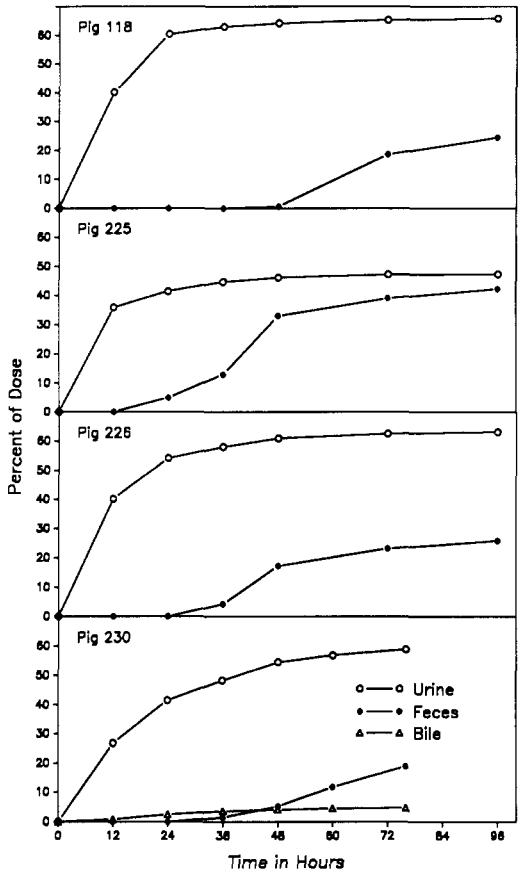

Figure 3. Cumulative excretion of ^{14}C after an oral dose of [^{14}C]-arsanilic acid. All pigs received a single oral dose of [^{14}C]arsanilic acid; however, before receiving the radioactive dose and during the collection period, pigs 226 and 230 received feed containing 0.01% arsanilic acid.

Table III. Concentration of ^{14}C in Swine Tissues from a Single Oral Dose of [^{14}C]Arsanilic Acid[a]

	ppb equivalent of arsanilic acid[b]			
tissue	pig 118	pig 225	pig 226	pig 230
liver	1323	1164	794	1513
kidney	205	117	132	545
lungs	85	63	52	220
adrenals	90	133		146
dermal fat	68	284	154	44
mesenteric fat	59	202	154	85
heart	48	26	36	139
muscle (from the round)	35	29	29	68
eviscerated carcass	49	26	29	78

[a] Time from dosing to sacrifice was 96 h for pigs 118, 225, and 226 and 76 h for pig 230. [b] Values are calculated by dividing dpm of $^{14}C/g$ of fresh tissue at time of sacrifice by the specific activity of the dose. See text for interpretation.

because there probably were nonspecific losses in these procedures. Analysis of urine from pig 226 is the primary basis for quantitative estimates of metabolites; however, the pattern was similar in urine of pig 225.

Metabolite 1 was characterized as arsanilic acid by comparing -FAB and NMR spectra with spectra from known arsanilic acid. We estimate [^{14}C]arsanilic acid comprised somewhere between 17% and 39% of the urinary ^{14}C metabolites. Metabolite 2 was characterized as *N*-acetylarsanilic acid by comparing -FAB and NMR spectra with spectra from known arsanilic acid reacted with acetic anhydride. We estimate *N*-acetylarsanilic acid comprised somewhere between 15% and 29% of the urinary ^{14}C metabolites.

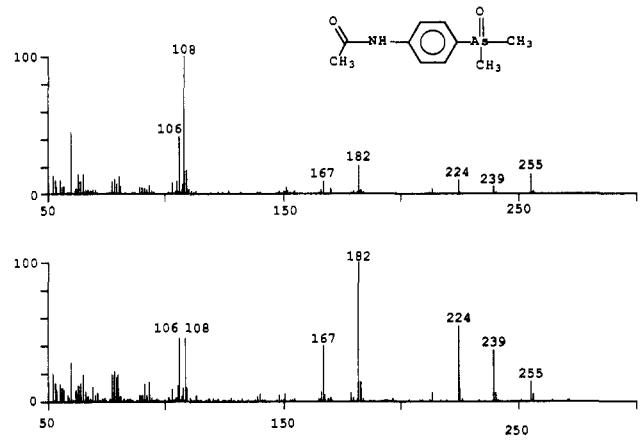

Figure 4. EI mass spectra of metabolite 3. Scans were taken 18 s apart.

Metabolite 3 was tentatively characterized as (4-acetamidophenyl)dimethylarsine oxide. Mass spectra (EI) of this metabolite are shown in Figure 4 (scans taken 18 s apart). Considerable change in the ratios of the apparent molecular ion and the ion at M - 16 in successive scans suggests either a mixture of compounds or decomposition on the probe. The measured mass (peak match) of the apparent molecular ion was within 2.5 ppm of the calculated mass for $C_{10}H_{14}AsNO_2$, the elemental composition of the structure shown in Figure 3 (resolution of the instrument is approximately 2.5 ppm). The measured mass of the peak at nominal mass 239 was 239.027 179. The calculated mass for $C_{10}H_{14}AsNO$ (a loss of oxygen from the apparent molecular ion) is 239.029 139, a difference of 8 ppm. No other reasonable elemental composition had a mass close to that observed.

The NMR spectrum of metabolite 3 showed singlets at δ 1.94 and 2.15 and doublets at δ 7.73 and 7.81 (J = 8.82 Hz). The peak at δ 1.94 compares favorably with methyl absorptions for dimethylarsenic acid at δ 1.97 (Sattler) and for dimethylphenylarsine oxide at δ 1.97. The spectrum of the metabolite also showed small peaks at δ 1.28 and 1.90. The peak at δ 1.28 is similar to the absorptions for dimethylphenylarsine at δ 1.15; however, the peak at δ 1.90 could not be assigned.

On the basis of the above evidence we tentatively assigned metabolite 3 the structure shown in Figure 4. At times the metabolite exhibited erratic behavior during the final HPLC step. A radioactive peak was sometimes observed during the reverse gradient after an injection. The mass spectrum of material in this peak was similar to that exhibited by the material eluted during the gradient from methanol to water. We have no explanation for this behavior. No attempt was made to isolate metabolite 3 from urine of pigs that had not received unlabeled arsanilic acid in their diet before and after the [^{14}C]arsanilic acid dose.

Figure 2 indicates two discrete fractions that were not characterized. One of these (10% of the urinary ^{14}C) was extremely polar, eluting with the void volume from the Porapak gradient chromatography system. We have no suggestion as to the chemical nature of this radioactivity. The other uncharacterized fraction (1% of the urinary ^{14}C) eluted from the Porapak gradient system at a methanol concentration similar to that which eluted the *N*-acetylarsanilic acid metabolite. The small amount of ^{14}C relative to the mass in this fraction prevented confirmation of this possibility.

Attempts to isolate ^{14}C metabolites from feces were unsuccessful. Extraction with methanol (3×) followed by

Table IV. Distribution of ^{14}C 72 h after an Oral Dose of [^{14}C]Arsanilic Acid to Roosters[a]

	% of dose[b]	
	colostomized	normal
urine	63.4 ± 4.6	
feces	26.6 ± 1.6	
excreta		87.0 ± 2.8
GI tract	1.5 ± 1.9	0.2 ± 0.1
carcass	0.4 ± 0.1	0.3 ± 0.1
total recovery	91.8 ± 1.1	87.5 ± 2.9

[a] Each bird received 11.45 mg (4.6×10^6 dpm) of [^{14}C]arsanilic acid. [b] Means ± SD, $N = 3$.

water (3×) solubilized 25% of the ^{14}C in the feces. This procedure also solubilized approximately 25% of the total mass in the feces. Porapak Q (as used with urine) was not useful in isolating radiolabeled metabolites from the methanol extract of the feces. Essentially no ^{14}C was extracted with less polar solvents such as ethyl acetate, amyl alcohol, or dichloromethane. It is also of note that after [^{14}C]arsanilic acid (dosing solution) was mixed, with normal feces, only 25% of the ^{14}C could be recovered by extraction with methanol and water (as described above).

Roosters. Table IV presents the distribution of ^{14}C 72 h after a dose of [^{14}C]arsanilic acid to roosters. As in the pig, the urine was the principal route of excretion. Excretion is very rapid with nearly 70% in the excreta by 6 h after dosing of the normal birds. Within 6 h after dosing of colostomized birds, approximately 10% and 45% were excreted in the feces and urine, respectively. Fecal excretion by colostomized birds may have been slightly slower than that for normal birds. Expressed as ppb equivalents of arsanilic acid in fresh tissue, the concentration of ^{14}C in liver and kidney of normal birds was 194 ± 62 and 111 ± 30, respectively; in colostomized birds these values were 417 ± 257 and 118 ± 43 (average of three birds ± SD). The concentration of ^{14}C in the ground carcass was too low to be measured reliably with the procedures used. A concentration of 50 dpm/g of fresh tissue (107 ppb arsanilic acid equiv) results in a count rate in the assay system of approximately twice background, and this is regarded as the lower limit of reliability.

Only one ^{14}C metabolite could be isolated from the rooster urine by using chromatographic systems similar to those used for swine urine (Porapak Q columns with pH adjusted to 4.5 in the initial column, Radial Pak and C-18 analytical). The metabolite was characterized by -FAB and NMR as unchanged arsanilic acid. The procedures used for this isolation did not suggest the presence of other ^{14}C compounds. No attempts were made to isolate radiolabeled metabolites from feces of colostomized birds or from excreta of normal birds.

Although arsanilic acid has been used in animal production for many years, the metabolic fate and mode of action remain obscure, and information on these subjects is sometimes contradictory. With respect to biotransformations in chickens, the data presented here agree with those previously reported (Moody and Williams, 1964; Overby and Straube, 1965). However, our data demonstrate extensive absorption from the gut of chickens, contrary to the suggestions of Moody and Williams (1964) (on the basis of theoretical considerations).

With respect to pigs, a previous study indicated arsanilic acid could not be detected in urine (Overby and Frost, 1960), whereas we found arsanilic acid to be the predominant urinary metabolite. This is not surprising considering the earlier studies were done without the aid of a radioactive label and the polar nature of arsanilic acid makes isolation difficult. *N*-Acetylarsanilic acid is a logical metabolite but had not been previously isolated. The (4-acetamidophenyl)dimethylarsine oxide was not an anticipated metabolite, and its mechanism of formation is not known.

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Registry No. Arsanilic acid, 98-50-0; *N*-acetylarsanilic acid, 618-22-4; (4-acetamido)dimethylarsine oxide, 129966-85-4.