Tway, Wood, Downing

J. Agric. Food Chem., Vol. 27, No. 4, 1979 **753**

Shafik, T., Bradway, D. E., Ehos, H. F., Yobs, A. R., *J. Agric. Food Chem.* **21**, 625 (1973).

Shafik, T. M., Peoples, A., "Benzyl Derivatives of Urinary Alkyl Phosphates", 172nd National Meeting of the American Chemical Society, Division of Pesticide Chemistry, San Francisco, CA, 1976.

St. John, L. E., Lisk, D. J., *J. Agric. Food Chem.* **16**, 48 (1968).

Watts, R. R., *J. Assoc. Off. Anal. Chem.* **48**, 1161 (1965).

White, E. H., Scherrer, H., *Tetrahedron Lett.* **21**, 758 (1961).

White, E. H., Baum, A. A., Eitel, D. E., *Org. Synth.* **48**, 102 (1968).

White, E. J., Maskill, H., Woodcock, D. J., Schroeder, M. A., *Tetrahedron Lett.* **21**, 1713 (1969).

Zollinger, H., "Azo and Diazo Chemistry, Aliphatic and Aromatic Compounds", Interscience, New York, 1961, p 122.

Determination of Arprinocid in Chicken Tissues by Gas Chromatography–Mass Spectrometry

Patricia Cala Tway,* James S. Wood, Jr., and George V. Downing

A procedure has been developed for the isolation and determination of the coccidiostat arprinocid and its metabolites in tissue. Analysis can be performed with a sensitivity of 0.04 ppm using a combined gas chromatographic–mass spectroscopic detection system. Recoveries of arprinocid spikes average 76% for liver, kidney, and muscle and 70% for skin/fat.

Arprinocid [6-amino-9-(2-chloro-6-fluorobenzyl)purine] (Figure 1) is a newly developed coccidiostat which is used prophylactically in broiler chickens. The drug is effective at the 70 ppm level against all important economic strains of coccidia and has been tested against more than 50 strains of *Eimeria tenella* and *Eimeria acervulina* (Miller et al., 1977; Tamas et al., 1978; Olson et al., 1978).

To study the tissue distribution of the arprinocid residue in chickens and to satisfy the requirements of governmental regulatory agencies, a sensitive, specific, and reliable chemical assay for the parent drug and its major drug-related metabolites was needed. Metabolism studies using radioactive tracer have shown that the largest and most persistent drug residues are found in the liver. While on-drug liver residue is primarily the parent compound, the residue in livers from chickens that are withdrawn from the drug for 5 days is only approximately 5% arprinocid. Most of the drug residue at this withdrawal time is covalently bonded to macromolecular constitutents of the tissue and is not easily solvent extractable from the tissue. It was found that acid hydrolysis of the tissue released approximately 70% of the radioactive residue; the acid hydrolyzes the residue, including any arprinocid, to form 2-choro-6-fluorobenzylamine. On the basis of this metabolism work (Carlin et al., 1976), liver was chosen as the target tissue and 2-chloro-6-fluorobenzylamine formed by acid hydrolysis as the marker substance. Both the use of liver as the target tissue and fluorochlorobenzylamine as the marker substance fulfill the requirements of the Food and Drug Administration for an acceptable animal drug residue assay (Federal Register, Feb 22, 1977).

Many different analytical techniques have been used to determine amines including fluorescence measurement after derivatization (Rinde and Troll, 1976; Sturgeon and Schulman, 1975), liquid chromatography using UV detection (Majors, 1973), and gas chromatography using electron-capture detection (Cala et al., 1972). Recently workers have used gas chromatography (GC) coupled with mass spectrometry (MS) as the detection system to quantitate low-level drug residues in biological extracts, primarily in plasma and urine (Summons et al., 1974; Narasimhachari et al., 1978; Carrington and Frigerio, 1977).

During the early work it was found that dansylation of the 2-chloro-6-fluorobenzylamine was quantitative (Carlin et al., 1976), and the product (Figure 1) could be manipulated without the losses due to volatility that were likely with the underivatized amine. It could also be determined by gas chromatography using electron-capture detection. However, initial experiments on arprinocid showed that gas chromatography with electron-capture detection did not have the necessary specificity for an assay with the desired sensitivity. A material was found to extract from the tissue which interfered with the dansyl derivative upon chromatography, and no combination of chromatographic conditions or extraction/purification steps were found to eliminate this interference. However, GC/MS using specific ion monitoring was found to provide the necessary sensitivity and specificity.

The procedure developed utilizes gas chromatography–mass spectrometry in the chemical ionization mode as the method of separation and detection. The combined drug residue is hydrolyzed to yield 2-chloro-6-fluorobenzylamine which is extracted from the tissue, dansylated, and cleaned up further through a series of extractions, and finally quantitated by GC/MS. The assay has a sensitivity of 0.04 ppm and a limit of detection of approximately 0.01 ppm. Recoveries of arprinocid spikes averaged 76% for liver, kidney, and muscle and 70% for skin/fat.

EXPERIMENTAL SECTION

Reagents. All organic solvents were nanograde quality. All other reagents were of analytical grade purity. The 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride) was reagent grade from Matheson, Coleman, & Bell. The dansyl derivative of 2-chloro-6-fluorobenzylamine was obtained from Merck Sharp & Dohme Research Laboratories, Rahway, NJ. All aqueous solutions were prepared with water which had been doubly distilled.

A standard solution of 2-chloro-6-fluorobenzylamine dansyl derivative was prepared by dissolving 1 mg of the

---

Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065.
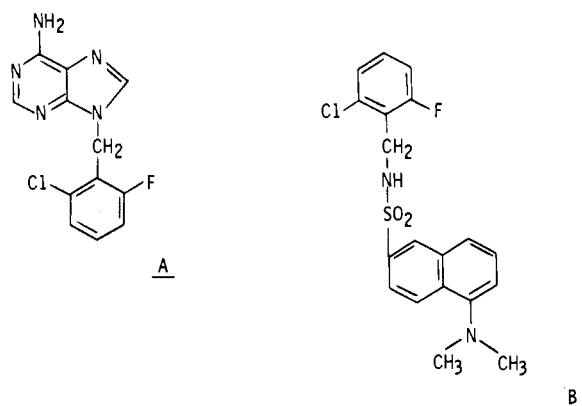
Figure 1. Structure of arprinocid (A) and the dansyl derivative of 2-chloro-6-fluorobenzylamine (B).

reference standard in 10 mL of methanol. This standard was diluted to appropriate levels (2, 1, 0.4 μg/mL) for use in calibration. The standards were stored in the refrigerator.

Apparatus. A Finnigan 3200 mass spectrometer with a CI head was used in conjunction with the manufacturer's Model 9500 gas chromatograph and a Finnigan model 6000 data system. The glass column (150 cm × 2 mm i.d.) was packed with 3% SP-2100 on 80–100 mesh Supelcon AW-DMCS (Supelco, Inc.) and conditioned at 260 °C with helium flow overnight. The operating conditions were as follows: column temperature, 245 °C, injector port, separator oven, and transfer line temperatures, 250 °C. The carrier gas (methane) pressure was set to give an ion source pressure of 900–1000 μm. Typical ion source operating conditions were: ion energy, 8.0 V; ion repeller 0 V; lens, –25 V; electron energy, 150 V; filament emission 0.9 mA. The voltage across the continuous dynode electron multiplier was –1900 V. The preamplifier sensitivity was 10^{-9} amp/V and the filter was 3 × 1 amu/s. The mass spectrometer was run in the multiple ion detection mode (MID), monitoring m/e 393 (M + 1 for B in Figure 1), m/e 395 (^{37}Cl isotope of M + 1), and m/e 421 (M + 29 or M + $C_2H_5^+$) ion arising from the methane used for chemical ionization.

Procedure. A flow diagram for the assay procedure is shown in Figure 2. Two grams of tissue are homogenized with 10 mL of 6 N hydrochloric acid in a Sorvall omni-mixer. The sample is transferred to a 250-mL Erlenmeyer flask, and the homogenizer cup is washed out with 10 mL of 6 N hydrochloric acid which is added to the sample. The sample is autoclaved at 120 °C for 15–16 h. The sample is transferred to a 50-mL centrifuge tube, made alkaline with 50% sodium hydroxide, and extracted three times with 10 mL of toluene each time. The toluene extracts are combined in another 50-mL centrifuge tube, and the hydrolysis product is extracted from the toluene three times with 5 mL of 1 N hydrochloric acid. The acid extracts are combined in a 250-mL round-bottom flask and evaporated to dryness on a rotary evaporator.

The residue is quantitatively transferred with methanol to a 15-mL centrifuge tube and evaporated to dryness in a warm water bath under a stream of nitrogen. Three milliliters of acetonitrile and 2 mL of hexane are added to the centrifuge tube. The sample is simultaneously ultrasonicated and shaken by hand for 4 min and centrifuged, and the acetonitrile is transferred to another centrifuge tube. The hexane layer is extracted with an additional 2 mL of acetonitrile, and the acetonitrile extracts are combined and evaporated to dryness.

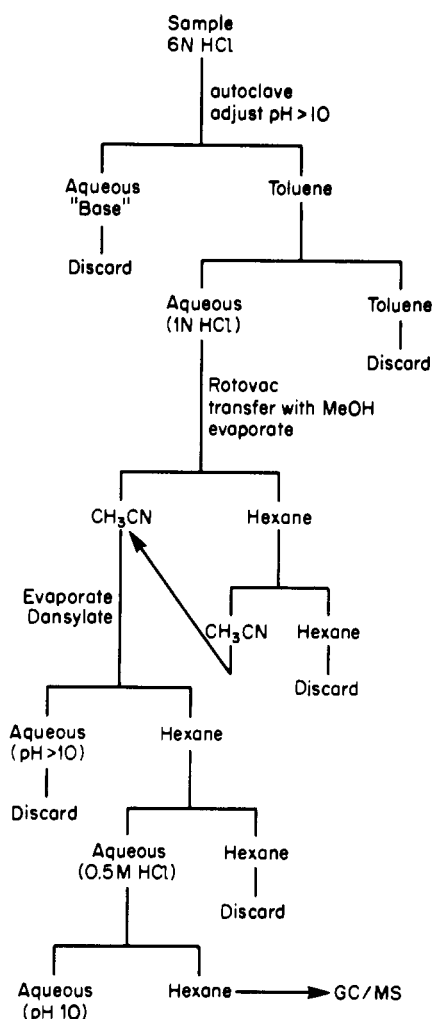The residue is dissolved in 2 mL of 0.2 M pH 8.8 bicarbonate buffer and 1 mL of dansyl chloride (1.0 g in 100

Figure 2. Flow diagram for the cleanup of arprinocid tissue residue samples.

mL of acetone), stoppered, and reacted in a water bath at 53–58 °C for 20 min. The samples are evaporated to 2.0 mL, made alkaline (pH >10) with 2 M sodium hydroxide, and extracted three times with 3 mL of hexane each time. The hexane extracts are combined and extracted twice with 2 mL of 0.5 M hydrochloric acid. The acid extract is made alkaline with 1 mL of 2 M sodium hydroxide and 2 mL of 2 M pH 10 carbonate buffer and extracted twice with 5 mL of hexane. The hexane is evaporated to dryness. The sample is split exactly in half by pipetting 4 mL of ethyl acetate into the tube, mixing thoroughly, and pipetting 2 mL from that tube into another centrifuge tube. Both portions are evaporated to dryness.

The residue in one of the sample tubes is dissolved in 200 μL of ethyl acetate and a 4-μL sample injected into the GC/MS. The other portion of the sample is dissolved in 200 μL of a standard solution of the fluorochloro-benzylamine dansyl derivative containing approximately the same concentration as estimated in the sample and 4 μL is similarly injected. The concentration of the sample is roughly estimated from the chromatogram of the unspiked sample, and a standard solution of about the same concentration is used to dissolve the second or spiked portion of the sample. For example, if the sample is estimated to contain 0.2–0.3 ppm arprinocid (0.2–0.3 μg/g × 2 g = 0.4–0.6 μg), each tube should contain approximately 0.25 μg of arprinocid equivalent which is equal on a molar basis to 0.35 μg of fluorochlorobenzylamine dansyl derivative. The sample should then be spiked with ap-

Tway, Wood, Downing

J. Agric. Food Chem., Vol. 27, No. 4, 1979 **755**

Table I. Residue Results from 5-Day-Off Livers from Chickens Fed 70 ppm Arprinocid

bird	total radioact. res., ppm	drug-related residue		% radioact. found as drug-related res.
		GC/MS, ppm	radioact. res., ppm	
9015	0.24	0.18	0.129	75
9016	0.25	0.14	0.150	56
9017	0.33	0.19	0.178	58
9041	0.30	0.17	0.150	57
9042	0.33	0.13	0.130	39
9043	0.29	0.16	0.125	55
av		0.16	0.143	57

Figure 3. Electron impact (EI) spectra of the dansyl derivative of 2-chloro-6-fluorobenzylamine.

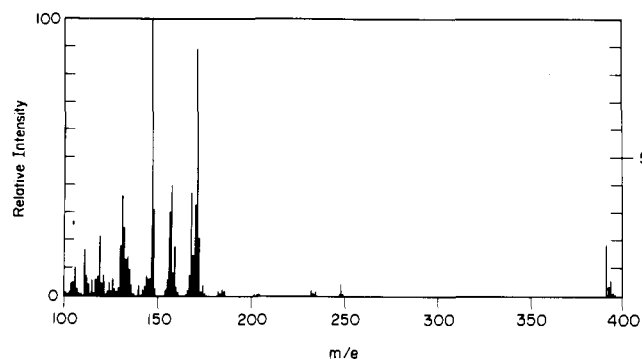**Figure 4.** Chemical ionization (CI) spectra of the dansyl derivative of 2-chloro-6-fluorobenzylamine.

proximately 0.35 μg of the dansyl derivative.

The area of the peaks from the MID computer run is measured by an autopeak search routine which is part of the software of the Finnigan 6000 data system. The arprinocid residue in a sample is calculated by comparing the peak area of the m/e 393 peak of the sample to the increment of area obtained on the spiked sample. The equation for calculating drug residue is

$$\text{residue (ppm)} = (A_{SA} \times \mu g_{SP})/(A_{SP} - A_{SA}) \quad (1)$$

where A_{SA} = peak area of sample, A_{SP} = peak area of spiked sample, and μg_{SP} = microgram arprinocid equivalent of spike.

RESULTS AND DISCUSSION

Since the tissue samples could not be cleaned up sufficiently to use electron-capture detection, the only other available detector with adequate sensitivity was a mass spectrometer. The electron impact (EI) and chemical ionization (CI) spectra of the dansyl derivative of fluorochlorobenzylamine are presented in Figures 3 and 4, respectively. Although the molecular ion in the EI mode was much weaker relative to lower mass fragments, the two methods had comparable sensitivities because of the greater efficiency of ionization in the EI mode. Chemical ionization was chosen because we felt it would be more specific, i.e., would be less likely to have ions arising from other tissue components, and it gave more ions at a high m/e value that could be used to confirm the identity of the drug residue.

The 2-chloro-6-fluorobenzylamine dansyl derivative has a molecular weight of 392. Chemical ionization using methane normally gives a protonated molecular ion at (M + 1), and cluster ions at (M + 29) formed from the compound and a $C_2H_5^+$ reactant species, and at (M + 41) formed from the compound and a $C_3H_5^+$ reactant species. With this compound an (M + 3) ion was seen which was the ^{37}Cl isotope of the protonated molecular ion. Three ions were monitored in this assay—m/e 393 (M + 1), 395 (M + 3), 421 (M + 29). The relative ratio of these three ions is 1:0.4:0.15. Only if a detector response is seen at the correct retention time of the compound and the ion ratio
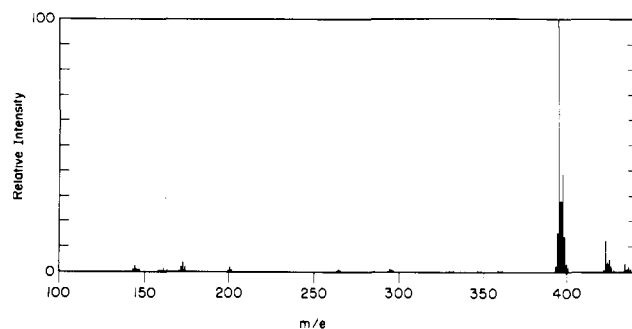
is approximately correct is the tissue determined to have a residue which is drug related. Quantitation is done using only the m/e 393 ion, but the other two ions are used to confirm the presence of a drug residue. It is impossible to use an ^{15}N or deuterated arprinocid internal standard because there is overlap between the ^{13}C isotope peaks and the Cl isotope cluster.

An increased area response was found for the dansyl derivative standard in the presence of a tissue extract, probably because there is less loss of the compound due to adsorption onto the column and the glass connecting lines to the mass spectrometer. It is postulated that extraneous material from the tissue extract essentially coats any active sites and lessens adsorption. Since the derivative responds differently in the presence and absence of tissue extract, the method of additions has been used for quantitation (Willard et al., 1965). The linearity of the method of additions was investigated by using samples diluted with one times and two times standard solutions. All such samples gave linear responses so that a single addition was chosen for simplicity.

Nonmedicated chicken muscle, skin/fat, liver, and kidney spiked with arprinocid at levels of 0.1–1.0 ppm and taken through the assay procedure gave recoveries of 60–90%. The recoveries averaged 76% for muscle, liver, and kidney and 70% for skin/fat with a standard deviation of 8%. Liver samples spiked with radioactive arprinocid and carried through the assay procedure also gave recoveries by radioactivity of 70–80%. Typical chromatograms can be seen in Figure 5. Figure 5A shows a blank liver, and Figure 5B shows a liver spiked with 0.25 ppm arprinocid and carried through the assay procedure. The printout automatically normalizes on the largest peak in a sample, so one must compare the signal-to-noise ratio of the same ion in different samples to obtain a relative idea of peak intensity. All control tissues tested had no detectable residue.

Tissue Residue Studies. Two residue studies were carried out on chickens medicated at 70 ppm with arprinocid in the feed. In the first study the chickens were fed ^{14}C-labeled arprinocid at 70 ppm for 44 days and then withdrawn from drug for 5 days before being sacrificed.
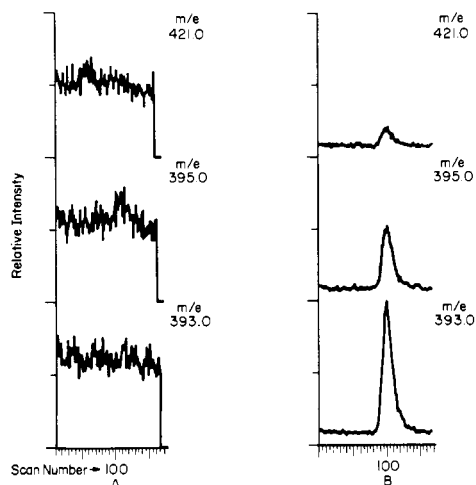
Figure 5. Multiple ion detection chromatograms of chicken liver samples: (A) nonmedicated liver, (B) liver spiked with 0.25 ppm arprinocid.

Table II. Arprinocid Residue Results Chickens Fed 70 ppm Arprinocid

	muscle,[a] ppm	skin/fat, ppm	kidney, ppm	liver, ppm
6 hours off	0.08	0.05	0.57	0.99
1 day off	<0.04[b]	<0.04	0.15	0.39
3 days off	<0.04	<0.04	0.10	0.29
5 days off			0.07	0.17
7 days off			<0.04	0.11
9 days off				0.08

[a] Each value is an average of the residue results from six chickens. [b] The sensitivity of the assay is 0.04 ppm. Any residue less than 0.04 ppm is not quantitated.

Only the liver samples were assayed. The results are present in Table I. Column 2 contains the total radioactive residue found in the tissue by combustion. Column 3 contains the drug related residue obtained by the GC/MS method. An aliquot of the solution which was injected into the GC/MS was also counted for total radioactivity, and column 4 shows the radioactivity in ppm drug related residue in the solution. The excellent agreement between columns 3 and 4 demonstrates the specificity of the GC/MS assay. Column 5 contains the percent of the total radioactive residue which was found as drug-related residue by GC/MS. The percent recovery averaged 57% which is close to what is to be expected from the 70% of the residue which is felt to be drug related and 76% recovery of arprinocid spikes through the assay procedure (0.70 × 0.76 = 0.53 or 53%).

The second residue study consisted of seven groups of

chickens (three males and three females per group). One group served to furnish nonmedicated control tissues, and the remaining groups received 70 ppm arprinocid in the feed for 44 days. One group of medicated chickens was sacrificed at each of 0, 1, 3, 5, 7, and 9 days off drug, and their tissues were assayed. The results are summarized in Table II.

Residues were found in on-drug muscle (<0.04–0.12 ppm), skin/fat (<0.04–0.08 ppm), kidney (0.31–0.96 ppm), and liver (0.68–1.64 ppm). At 1 day off drug, only liver and kidney contained drug residues (average 0.39 and 0.15 ppm, respectively). By 7 days off drug, only liver had a detectable residue, averaging 0.11 ppm. Starting with the first day, the liver residue data showed a linear semilogarithmic decay curve with a half-life of 3.5 days. All the control tissues had no detectable residue.

LITERATURE CITED

Cala, P. C., Trenner, N. R., Buhs, R. P., Downing, G. V., Jr., Smith, J. L., VandenHeuvel, W. J. A., *J. Agric. Food Chem.* **20**, 337 (1972).
Carlin, J., Jacob, T. A., Wolf, F. J., private communication, 1976.
Carrington, R., Frigerio, A., *Drug Metab. Rev.* **6**, 243 (1977).
Federal Register **42**(35), Feb 22, 1977.
Majors, R. E., *Anal. Chem.* **45**, 755 (1973).
Miller, B. M., McManus, E. C., Olson, G., Schleim, K. D., Van Iderstine, A. A., Graham, D. W., Brown, J. E., Rogers, E. F., *Poultry Sci.* **56**, 2039 (1977).
Narasimhachari, N., Prakash, U., Helgeson, E., Davis, J. M., *J. Chromatogr. Sci.* **16**, 263 (1978).
Olson, G., Tamas, T., Smith, D. A., Weppelman, R. M., Schleim, K., McManus, E. C., *Poultry Sci.* **57**, 1245 (1978).
Rinde, E., Troll, W., *Anal. Chem.* **48**, 542 (1976).
Sturgeon, R. J., Schulman, S. G., *Anal. Chim. Acta* **75**, 225 (1975).
Summons, R. E., Pereira, W. E., Reynolds, W. E., Rindfleisch, T. C., Duffield, A. M., *Anal. Chem.* **46**, 582 (1974).
Tamas, T., Olson, G., Smith, D. A., Miller, B. M., *Poultry Sci.* **57**, 381 (1978).
Willard, H. H., Merritt, L. L., Jr., Dean, J. A., "Instrumental Methods of Analysis", Van Nostrand, New York, 1965, pp 342–344.