

DRUG RESIDUES IN ANIMAL TISSUES

Screening Tests for Sulfa Drugs and/or Dinitrobenzamide Coccidiostats and Their Monoamino Metabolites in Chicken Livers

OWEN W. PARKS

U.S. Department of Agriculture, Eastern Regional Research Center, Philadelphia, PA 19118

Two procedures were developed for the simultaneous determination of 0.1 ppm sulfaquinoxaline and sulfadimethoxine, 1.0 ppm Zoalene and nitromide, and/or 0.1 ppm of their reduced coccidiostat metabolites from the same sample of chicken liver. Both methods include blender extraction of 5 g liver with chloroform-ethyl acetate (1 + 1), adsorption of the drugs and metabolites on neutral alumina, and subsequent elution with 0.2M carbonate buffer (pH 11.0). In Method A, all parent drugs and coccidiostat metabolites were partitioned into dichloromethane, following the addition of a small amount of tetrabutylammonium hydroxide (TBAH). The presence of the dinitrobenzamide was confirmed by the formation of a color with TBAH, which occurs when the solvent is concentrated (Zoalene = green; nitromide = red). Sulfa drugs and coccidiostat metabolites were detected by the Bratton-Marshall reaction after thin layer chromatographic (TLC) separation. Method B separates the individual classes by selective extraction techniques. The coccidiostats and their metabolites were extracted from the buffer eluate by ethyl acetate-dichloromethane (3 + 1) before ion pairing: sulfa drugs were extracted with dichloromethane after ion pairing with TBAH. The detection techniques were similar to those described for Method A.

A wide variety of drugs (1) are available for the prevention and control of caecal and intestinal coccidiosis in chickens. Among the drugs are aklomide, 2-chloro-4-nitrobenzamide, and the dinitro-substituted compounds nitromide (3,5-dinitrobenzamidé) and Zoalene (3,5-dinitro-*o*-toluamide)—all of which are considered to be potentially toxic to humans because they contain nitro-groups. Although analytical methods are available for detecting and quantitating the individual drugs in chicken tissues (2), the procedures are generally time consuming and cumbersome. As a result, few data have been obtained to determine the extent of the residue occurrence in animal tissues caused by the use of these drugs. The need for a rapid, simple screening procedure for this purpose is evident.

The coccidiostats are readily metabolized (3) in vivo to monoamino compounds, which are included in the violative levels established by the regulatory agencies for chicken livers (4). The procedure for determining Zoalene residues does not determine its metabolites, which require a separate method (5). However, the latter method along with the analytical procedures for aklomide, nitromide, and their metabolites relies on the Bratton-Marshall (B-M) reaction that is widely used for the colorimetric detection of sulfonamides in animal feeds and tissue. Sulfaquinoxaline, and to a lesser extent sulfadimethoxine, are also fed to chickens to prevent coccidiosis and certain microbial diseases. Hence, the use of the B-M reagent creates the potential for the erroneous determination of sulfa drugs in the presence of coccidiostat metabolites in chicken tissues and vice versa. The screening methods presented here were designed to overcome this problem.

METHOD

Reagent and Materials

(a) *Solvents*.—Ethyl acetate, hexane, dichloromethane, methanol, and acetonitrile (Distilled-in-Glass®, Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442). Chloroform, Baker Analyzed reagent (J. T. Baker Chemical Co., Phillipsburg, NJ 08665). *N,N*-Dimethylformamide (Aldrich Chemical Co., Inc., Milwaukee, WI 53233).

(b) *Tetrabutylammonium hydroxide (TBAH)*—40% aqueous solution (Aldrich Chemical Co., Inc.).

(c) *0.2M and 0.5M carbonate buffer*.—pH 11. Prepare from 0.2M and 0.5M solutions of sodium carbonate and sodium bicarbonate.

(d) *Pipet tip*.—5 mL (Rainin Instrument Co., Woburn, MA 01801).

(e) *Neutral alumina*.—Brockman Activity I, 80–200 mesh (Fisher Scientific Co., King of Prussia, PA). Insert 5 mm glass bead into 5 mL pipet tip. Layer glass bead with 0.5 cm sea sand followed by 3 cm bed of neutral alumina—packed firmly by gently tapping top of pipet tip. Add 0.25 cm layer of sea sand. Wash column with three 2 mL portions of CHCl₃-ethyl acetate (1 + 1) before use.

(f) *Sulfa drugs*.—Sulfadimethoxine (Hoffmann-La Roche, Inc., Nutley, NJ 07110); sulfaquinoxaline (Pfaltz and Bauer, Inc., Stamford, CT 06902).

(g) *Aklomide; nitromide; Zoalene*.—Salsbury Laboratories, Charles City, IA 50616.

(h) *3-Amino-5-nitro-*o*-toluamide (3-ANOT)*.—Gift from Dow Chemical USA, Midland, MI 48640. 3-Nitro-5-amino-*o*-toluamide (5-ANOT), 2-chloro-4-aminobenzamide, and 3-amino-5-nitrobenzamide—see acknowledgments.

Apparatus

(a) *Centrifuges*.—Sorvall Superspeed centrifuge, Type SS-1 rotor (Ivan Sorvall, Inc., Norwalk, CT). International Clinical centrifuge, rotor No. 273 (International Equipment Co., Needham Heights, MA 02194).

(b) *Vortex stirrer*.—Super Mixer (Lab-Line Instruments, Inc., Melrose Park, IL 60160).

(c) *Tissue grinder*.—Brinkmann Polytron® homogenizer (Brinkmann Instruments Inc., Westbury, NY 11590).

(d) *Liquid chromatography (LC)*.—Altex Model 100A pump (Altex Scientific Inc., Berkeley, CA 94710) connected to Schoeffel Model SF770 Spectroflow variable wavelength detector operated at 254 nm. Altex Model 210 sampling valve with a 50 µL loop. Column—25 cm × 4.6 mm id, 5 µm Alltech C₁₈ (Alltech Assoc., Deerfield, IL 60015). Mobile phases: sulfa drugs—methanol-water-acetic acid (40 + 59.5 + 0.5); coccidiostats—methanol-water (30 + 70); coccidiostat metabolites—acetonitrile-water (10 + 90).

(e) *Thin layer chromatography (TLC)*.—2.5 cm × 10.0 cm glass plate with 250 µm layer of silica gel G (Analtech, Newark, DE 19711). Developing solvent—CHCl₃-ethyl acetate-methanol (5 + 5 + 1). Develop plates to 0.5 cm height and dry in forced air oven 1 min at 50°C. Redevelop plates to

height of 1.0 cm, then 4.0 cm, with oven drying between and after attaining final height. Visualize sulfa drugs and coccidiostat metabolites with Bratton-Marshall (B-M) spray reagent according to procedure previously described (6). R_f values: sulfadimethoxine, 0.74; sulfaquinoxaline, 0.67; 2-chloro-4-aminobenzamide, 0.58; 3-amino-5-nitrobenzamide, 0.55; 3-ANOT, 0.56; and 5-ANOT, 0.45.

Analytical Procedure

Weigh 5 g frozen chicken liver, obtained from cross section of individual organ or from homogenized sample of livers, into 50 mL polypropylene centrifuge tube. Let sample partially thaw. Add 20 mL CHCl_3 -ethyl acetate (1 + 1) and blend 30 s with Polytron homogenizer at low speed. Centrifuge 5 min at 3000 rpm. Remove aqueous layer with disposable Pasteur pipet and discard. Recover solvent with disposable pipet and filter through 3 cm column of anhydrous sodium sulfate contained in large volume (4 mL) Pasteur pipet plugged at top and bottom with small wad of glass wool. Collect 10 mL filtrate. Pass filtrate through neutral alumina column. Wash column with 7 mL CHCl_3 . Remove excess CHCl_3 from column with air pressure and continue pressure until column dries as evidenced by disappearance of moisture on outside of column. Elute column with 0.2M, pH 11 carbonate buffer, using air pressure if necessary to obtain flow of 1–2 mL/min. Collect first 5 mL eluate in 15 mL screw-cap centrifuge tube. Add 5 mL hexane and shake carefully 1 min, using rocking motion. Centrifuge 2 min at 2500 rpm. Remove hexane with disposable pipet and discard. Proceed to Method A or Method B.

Method A—Simultaneous Detection of Sulfa Drugs and Dinitrobenzamide Coccidiostats and Their Monoamino Metabolites

Add 30 μL TBAH solution to eluate in centrifuge tube and vortex-mix 15 s. Add 10 mL dichloromethane and shake vigorously 2 min, using rocking motion. Centrifuge 2 min at 2500 rpm. Recover dichloromethane as previously described (6). Evaporate solvent in 9 mL screw-cap specimen vial at 40°C under stream of nitrogen to just dryness, noting color of residue. Definite green color indicates presence of Zoalene; red indicates nitromide. Dissolve residue in 0.2 mL methanol and spot 10 μL in 1.0–1.5 μL increments on TLC plates, drying spot with stream of nitrogen between applications. Subject samples to TLC and B-M spray reagent to visualize sulfa drugs and monoamino coccidiostat metabolites.

Method B—Separate Detection of Sulfa Drugs and Dinitrobenzamide Coccidiostats and Their Monoamino Metabolites

(a) *Monoaminobenzamide metabolites.*—Add 1 mL 0.5M, pH 11 buffer and 9 mL ethyl acetate-dichloromethane (3 + 1) to alumina eluate in centrifuge tube. Shake vigorously 2 min, using rocking motion. Centrifuge 2 min at 2500 rpm. Remove ethyl acetate-dichloromethane with disposable Pasteur pipet and evaporate to dryness in 9 mL screw-cap specimen vial at 50°C under stream of nitrogen. Dissolve residue in 0.2 mL methanol. Spot 10 μL solution and subject samples to TLC and B-M visualization reagents as described above.

(b) *Dinitrobenzamide coccidiostats.*—Evaporate TLC methanol solution in (a) above to dryness. Dissolve residue in 0.1 mL DMF. Add 1 drop of TBAH solution and note any color formation—green indicates presence of Zoalene; red indicates nitromide.

(c) *Sulfa drugs.*—Add 30 μL TBAH solution to ethyl acetate-dichloromethane extracted alumina eluate in (a) above.

Mix thoroughly. Add 9 mL dichloromethane and shake vigorously 2 min, using rocking motion. Centrifuge 2 min at 2500 rpm. Recover dichloromethane and evaporate to dryness at 50°C in 9 mL screw-cap specimen vial. Dissolve residue in 0.2 mL methanol. Subject sample to TLC and B-M visualization reagents according to procedure described above.

Recovery Studies

Fresh chicken livers were obtained locally and held frozen at -10°C . Five g samples of individual livers were spiked while frozen with methanol solutions of the drugs and/or metabolites (sulfaquinoxaline and sulfadimethoxine, 0.12 $\mu\text{g}/\mu\text{L}$; Zoalene and nitromide, 0.4 $\mu\text{g}/\mu\text{L}$; 3-ANOT and 5-ANOT, 0.18 $\mu\text{g}/\mu\text{L}$; and 2-chloro-4-aminobenzamide, 0.19 $\mu\text{g}/\mu\text{L}$). The spiked livers were held frozen for 1 h before extraction. Controls for the spiked samples were obtained from the same liver. Recovery data were determined by LC. To the residues obtained by Method A or B was added 0.4 mL of the appropriate mobile phase. The samples were vortex-mixed 30 s, followed by centrifugation 1 min at 2500 rpm. Fifty μL samples were injected onto the LC column. Recoveries were determined on the basis of peak heights relative to those of known quantities of the drugs and metabolites. TBAH was excluded from recovery studies of the coccidiostats by Method A because the dinitrobenzamides decomposed on evaporation. Difficulties in purifying 3-amino-5-nitrobenzamide prevented carrying out recovery studies on this metabolite. Retention times (min) were sulfadimethoxine, 12.8; sulfaquinoxaline, 15.2; Zoalene, 13.2; nitromide, 14.5; 3-ANOT, 11.9; 5-ANOT, 16.1; and 2-chloro-4-aminobenzamide, 8.5.

Results and Discussion

Preliminary studies revealed that Zoalene, nitromide, and aklomide were readily metabolized *in vitro* in chicken livers to their monoamino derivatives at room temperature. These observations were, generally, in keeping with previous reports (3, 7). Hence, screening procedures for detecting the coccidiostats in liver tissues must detect the *in vivo* metabolites and/or those that arise *in vitro* as the result of the unavoidable time lapse between sacrifice of the birds and freezing of the excised liver tissue. Because the methods of detecting the monoaminobenzamide metabolites are the same as those used for sulfa drugs (i.e., the B-M reaction), the screening procedure must differentiate these 2 classes of drugs. Method A differentiates the coccidiostat metabolites, sulfadimethoxine and sulfaquinoxaline, on the basis of TLC retention values; Method B separates the coccidiostat metabolites from the sulfa drugs before TLC separation and detection procedures by selective extraction techniques.

The parent dinitrobenzamide coccidiostats are detected in both methods by the formation of a color with TBAH at >1.0 ppm (liver basis). Aklomide, a mononitrobenzamide, does not form a color and is not detectable as the parent compound by the screening procedure. In Method A, TBAH acts initially as a counter ion to extract the sulfa drugs from the basic solution with dichloromethane. On evaporation of the solvent, the partitioned TBAH forms the color with the dinitrobenzamide coccidiostats. In actual practice, the concentrated solution takes on the appropriate color when approximately 0.5–1.0 mL solution containing >1.0 ppm coccidiostat remains. In Method B, at relatively high concentrations, the appropriate color is formed with DMF alone, but the color is greatly intensified by the addition of TBAH. In both procedures, control sample extracts exhibit only slight yellow colors in the presence of TBAH.

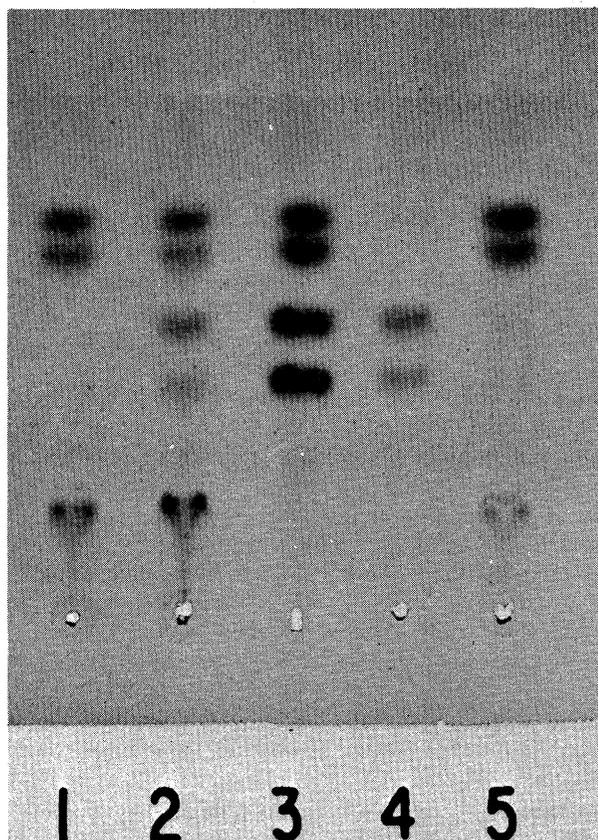


Figure 1. TLC of extracts of chicken livers treated according to Methods A and B. Livers spiked with 0.4 ppm sulfadimethoxine, 0.4 ppm sulfaquinolaxaline, and 1.0 ppm Zoalene before analyses. 1) Unabused liver, Method A. 2) Abused liver, Method A. 3) Authentic sulfadimethoxine; sulfaquinolaxaline; 3-ANOT; 5-ANOT (decreasing R_f). 4) Abused liver, Method B, coccidiostat fraction. 5) Abused liver, Method B, sulfa fraction. Abused liver—sample thawed and held 30 min at ambient temperature.

The monoaminobenzamide metabolites, sulfaquinolaxaline and sulfadimethoxine, are detected using the B-M spray reagent following TLC. In addition to detecting the Zoalene and nitromide metabolites, the procedures are capable of detecting 0.1 ppm 2-chloro-4-aminobenzamide. In contrast to previous reports (8), studies have shown that a second metabolite, tentatively identified as 5-ANOT on the basis of TLC and LC studies, is formed during the *in vitro* metabolism of Zoalene. This latter observation aids in differentiating the presence of Zoalene metabolites from the singular metabolites of nitromide and aklomide in liver tissue, despite similar R_f values. The separation of the sulfa drugs and coccidiostat metabolites into their respective classes by Method B is not absolute because approximately 10% of each class of drugs is carried over into the other class. The carryover does not, however, prevent recognition of the class of drugs present, and it can be avoided by additional extractions, if desired.

Figure 1 illustrates the TLC results of Methods A and B on chicken liver samples spiked with sulfadimethoxine, sulfaquinolaxaline, and Zoalene. Holding sample 1 at -10°C before using Method A resulted in the detection of the sulfa drugs. Holding sample 2 at room temperature 30 min before using Method A showed the additional presence of 3-ANOT and 5-ANOT. Samples 4 and 5, representing the coccidiostat and sulfa fractions obtained by using Method B on an abused liver, demonstrate the separation of classes by this procedure. The irregular (bottom) B-M positive spot in samples 1, 2, and 5 is anthranilic acid, a naturally occurring compound in liver tissues (9). The detection limits for sulfadimethoxine and sulfaquinolaxaline have been found to be <0.1 ppm. Although

Table 1. Recovery (%)^a of sulfa drugs, dinitrococcidiostats, and coccidiostat metabolites added to chicken livers

Sample	Added, ppm	Method A	Method B
Sulfadimethoxine	0.1, 0.2, 0.4	78.5 \pm 4.0	71.0 \pm 4.5
Sulfaquinolaxaline	0.1, 0.2, 0.4	63.9 \pm 5.5	62.3 \pm 8.8
Zoalene	1.0	78.2 \pm 1.4	79.4 \pm 3.1
3-ANOT	0.5	34.4 \pm 3.3	61.1 \pm 1.2
5-ANOT	0.5	17.2 \pm 1.7	38.6 \pm 2.7
Nitromide	1.0	60.2 \pm 2.6	66.5 \pm 5.7
2-Chloro-4-aminobenzamide	0.5	23.6 \pm 1.8	36.7 \pm 1.5

^aMean and standard deviation of 6 determinations at each concentration.

only these sulfa drugs have been used in this study, others should give the same results.

Table 1 summarizes the drug recoveries obtained by using Methods A and B on chicken livers spiked with varying concentrations of sulfa drugs, coccidiostats, and coccidiostat metabolites. Although the recoveries of the coccidiostat metabolites were low, no interferences were noted. Also, there was no difficulty detecting them at the 0.1 ppm level by TLC, partially as a result of the tight bands obtained. Attempts to improve recoveries of the metabolites by varying solvent systems resulted in lower recoveries and therefore higher detection limits, of the sulfa drugs.

The procedures were applied to livers of white leghorns that were fed a diet containing 0.0125% Zoalene for 5 weeks, and were sacrificed while still on the medicated feed. The livers were placed in dry ice within minutes of sacrificing the chickens. There was no difficulty detecting Zoalene by using either method on these samples. TLC of extracts obtained by either method revealed relatively large amounts of both the 3- and 5-ANOT. In addition, 3 minor *in vivo* metabolites were present in these extracts, the identities of which are unknown but currently under investigation. The minor metabolites, which may appear by using these methods, do not interfere with assessing the nature of the drug residues. The significance of minor metabolites is revealed in the fact that the 6.0 ppm violative level established for Zoalene in chicken livers (4) includes the parent drug plus the single metabolite, 3-ANOT. This study suggests that setting a violative level for a readily metabolized drug such as Zoalene without the ability to measure all principal metabolites may be unrealistic in determining whether a significant residue problem is present.

In conclusion, an analyst running 4 samples concurrently by Method A can complete the analyses in 2 h; Method B requires an additional 1/2 h for completion. Method B has the advantage of eliminating any doubt as to the class of compounds present in the tissues.

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