Gas Chromatographic Determination of Clopidol in Poultry Feeds

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A method is presented for the determination of clopidol (3,5-dichloro-2,6-dimethyl-4-pyridinol) added to poultry feeds at rates from 0.004 to 0.028%. The compound is extracted with methanolic ammonium hydroxide. After appropriate dilution with methanol, an aliquot of the extract is treated with ethereal diazomethane. The 3,5-dichloro-4-methoxy-2,6-lutidine produced is transferred to benzene and determined by gas chromatography.

Clopidol is the active ingredient in Coyden coccidiostat developed by The Dow Chemical Co. and is already in use as a medicant in poultry feeds. The development effort necessarily included devising a feed assay method suitable for both production control and regulatory uses.

Early effort resulted in a spectrophotometric method (Thiegs, 1964) which had the necessary precision but entailed a rather lengthy cleanup procedure. The proposed gas chromatographic method is simpler than the spectrophotometric procedure and is specific for clopidol in all feeds encountered to date.

ANALYTICAL METHOD

Reagents. Extraction solvent. Mixture of one volume of concentrated NH_4OH, ACS grade, and 19 volumes of methanol.

Diazald (Aldrich Chemical Co.).

Diazomethane reagent. Ethereal solution containing approximately 18 mg. per ml. (De Boer and Backer, 1963). Caution: Diazomethane is toxic, can cause specific sensitivity, and is potentially explosive. Do not breathe vapor or allow solutions to contact the body. Prepare the reagent in a hood. Avoid using etched glassware, ground glass joints, or glass tubing with sharp edges. Make all connections with rubber stoppers. Do not expose diazomethane solutions to direct sunlight or strong artificial light.

Clopidol and 3,5-dichloro-4-methoxy-2,6-lutidine, analytical standards (Agricultural Products Department, The Dow Chemical Co.).

Gas Chromatography. Chromatograph: Barber-Colman Model 10, equipped with Sr^{90} detector (Barber-Colman No. A-4148). Column: 6-foot × 3-mm. (i.d.) borosilicate glass U-tube, packed with 20% DC-200 fluid (12,500 cp.) on 80- to 100-mesh Chromosorb W, acid washed. Condition overnight at 200° C. Operating conditions: carrier gas, prepurified nitrogen (99.996%), 130 ml. per minute. Column temperature, 135° C. Detector temperature, 190° C. Flash heater temperature, 210° C. Polarizing voltage, adjust to obtain as linear a response as possible (usually 10 to 15 volts). Electrometer sensitivity, 10^{-9} amp. full scale, using a 5-mv. recorder. Injection technique: Fill the needle of a 10-μl. syringe with benzene. Carefully draw 3.0 μl. of sample into the syringe. Inject slowly (2 to 3 seconds).

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Procedure. Weigh 50.0 grams of feed into a 500-ml. volumetric flask. Add 250 ml. of methanolic ammonium hydroxide reagent and a stirring bar. Place on a magnetic stirrer and stir vigorously for 20 minutes. Remove the stirring bar and dilute to 500.0 ml. with methanol. Mix and allow to settle for 10 minutes. For feed samples containing from 0.008 to 0.016% clopidol, dilute 5.0 ml. of the supernatant to 100.0 ml. with methanol in a volumetric flask. Adjust the pH to 7.2 to 7.8 with concentrated HCl just before making the final volume adjustment. Pipet 1 ml. of the diluted extract into a 13 × 100 mm. screw-cap culture tube. (For feed samples containing less than 0.008% clopidol, dilute 5.0 ml. of the supernatant to 50.0 ml. instead of 100.0 ml. For samples containing more than 0.016% of the coccidiostat, pipet 0.5 ml. of the diluted extract into a culture tube and add 0.5 ml. of methanol.) Add 1 ml. of diazomethane reagent, cap tightly, (foil-lined cap) and place the lower one-fourth of the tube in a steam bath for 2 minutes. Remove from the steam bath and allow to cool for 5 minutes. Remove the cap, add a boiling chip, and boil down to 0.2 to 0.3 ml. on a steam bath. To prevent loss of the derivative, apply heat only to the bottom tip of the tube, keeping the upper portion cool enough to allow some refluxing of escaping solvents. Add 5 ml. of distilled water and 1.0 ml. of benzene. Shake the tube for 1 minute and centrifuge at about 1800 r.p.m. for 5 minutes. Inject 3.0 μl. of the benzene phase into the gas chromatographic column and determine the resulting peak height. Typical chromatograms for control feed and similar feed with 0.0125% clopidol added are shown in Figure 1.

Correction for Feed Volume. Fill a 100-ml. M.C.A. volumetric flask (Corning No. 5800) to the graduation mark with methanol. Add 10.0 grams of feed and allow

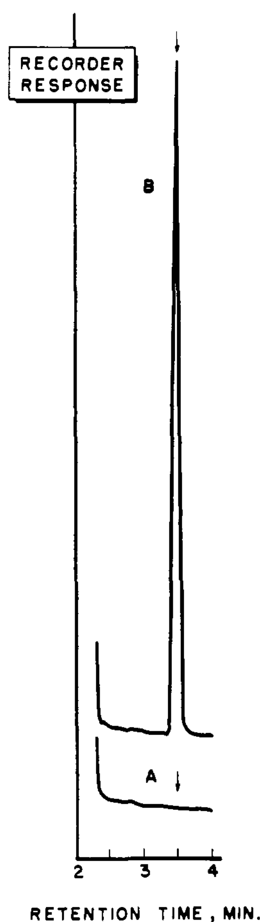

Figure 1. Representative chromatograms
Arrows indicate retention time of 3,5-dichloro-4-methoxy-2,6-lutidine. Curves were obtained from: *A*, control feed; *B*, feed containing 0.0125% clopidol

to settle for 10 minutes. Using a 10-ml. serological pipet, measure the volume of methanol above the graduation mark.

Preparation of Calibration Curve. Inject 3.0-μl. aliquots of appropriate 3,5-dichloro-4-methoxy-2,6-lutidine working standards (in benzene) into the chromatograph, covering the concentration range from 0.3 to 1.0 μg. per ml. (clopidol equivalent). Plot peak heights obtained *vs.* corresponding concentrations of clopidol and construct a calibration curve. A typical curve is shown in Figure 2.

Calculations. Determine, from the calibration curve, the concentration of clopidol in the final benzene solution.

% Clopidol = μg. per ml. found in benzene solution $\times$ $F \times (100 - V) \times 10^{-4}$

F = 1 for samples whose extracts were diluted 5 to 50 and 1 ml. taken for analysis.

F = 2 for samples whose extracts were diluted 5 to 100 and 1 ml. taken for analysis.

F = 4 for samples whose extracts were diluted 5 to 100 and 0.5 ml. taken for analysis.

V = volume of 10 grams of feed (ml.)

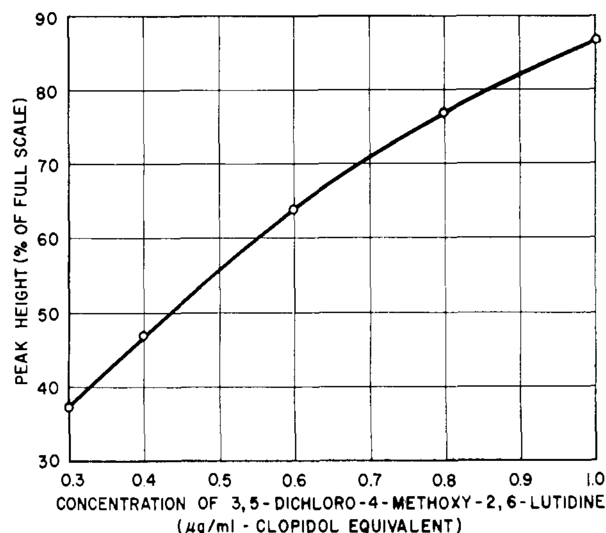

Figure 2. Calibration curve for gas chromatographic determination of clopidol in feed

RESULTS AND DISCUSSION

The response of the detector to 3,5-dichloro-4-methoxy-2,6-lutidine is affected by operating parameters, especially applied voltage and background components in the gas stream. That voltage should be selected which results in a response curve having the least deviation from linearity over the concentration range of interest. The background problem varies with different samples. Since the troublesome component is not detected and recorded, its effect is manifest only as a depression in detector response to 3,5-dichloro-4-methoxy-2,6-lutidine. Thus, when chromatographing feed extracts it is necessary to standardize frequently by injecting standards. If response becomes erratic, injection of extracts must be suspended until extraneous components are purged from the column and consistent response is restored.

A number of commercial feed samples from different geographic locations were analyzed and none showed a response at the retention time for the clopidol derivative. Thus, apparently no blank correction is necessary.

A comparison of three sampling methods, using feed prepared in a commercial mixer, is shown in Table I. The clopidol was added in the form of a 25% premix. The methods were: random 10-gram samples, 10-gram aliquots of a 100-gram sample which had been mixed for 1 minute in a Waring Blendor, and random 50-gram samples. Clearly, analysis of 50-gram samples, probably the practical limit for sample size, produced the most reliable results.

The efficiency of the proposed method was determined by adding known amounts of clopidol, in methanol solution, to 50-gram samples of feed and applying the procedure as described. (Note: The feed contained 50 grams of penicillin, 50 grams of zinc Bacitracin, and 45 grams of 3-nitro-4-hydroxyphenylarsonic acid per ton). The results, presented in Table II, show that recovery of clopidol added in this manner is quantitative.

Table I. Analysis of Feed Containing Clopidol Using Three Sampling Methods

Sample Size, Grams	Rep.	% Clopidol Found
		Sampling Method—Random
10	1	0.0120
	2	0.0155
	3	0.0136
	4	0.0119
	5	0.0138
	6	0.0137
	7	0.0121
	8	0.0115
		Average 0.0130 ± 0.0023[a]
		Sampling Method—Blended
10	1	0.0126
	2	0.0128
	3	0.0135
	4	0.0130
	5	0.0116
	6	0.0124
	7	0.0130
	8	0.0137
		Average 0.0128 ± 0.0014[a]
		Sampling Method—Random
50	1	0.0130
	2	0.0130
	3	0.0132
	4	0.0124
	5	0.0123
	6	0.0121
	7	0.0128
	8	0.0130
		Average 0.0127 ± 0.0008[a]

[a] 95% confidence.

To determine whether clopidol is extracted as readily from aged feed as it is from feed analyzed immediately after spiking, 750 mg. of 25% premix was added to 1500 grams of broiler feed in a 1-gallon jar equipped with mixing bar. After mixing for 5 hours by continuous rolling, the feed was allowed to stand in the closed jar at room temperature. Samples were analyzed at intervals over a period of 17 weeks. The results, in Table III, show no loss in efficiency of the method as the feed aged. This experiment was conducted concurrently with method development. Thus, only the final (17-week) samples were analyzed by the finalized method as described in this report. Ten-gram samples were employed in early analyses. This probably accounts for the raggedness of the data. There is no doubt, however, of the final conclusion that recovery of clopidol from feed does not decrease with aging.

Table II. Recovery of Clopidol Added to Feed

% Clopidol Added	% Clopidol Found	% Recovery
0.0040	0.00408	102
0.0040	0.00394	98.5
0.0080	0.00784	98
0.0080	0.00792	99
0.0120	0.01168	97.5
0.0120	0.01166	97.5
0.0200	0.02024	101
0.0200	0.02016	101
0.0280	0.02792	99.5
0.0280	0.02784	99.5
	Average	99.5

Table III. Recovery of Clopidol from Aged Feed[a]

Age of Feed, Days	% Clopidol Found	% Recovery
0	0.0117	93.5
13	0.0112	89.5
15	0.0116	93
27	0.0118	94.5
56	0.0114	91
119	0.0118	94.5

[a] 0.0125% clopidol added.

LITERATURE CITED

De Boer, T. J., Backer, H. J., "Organic Syntheses," Coll. Vol. IV, N. Rabjohn, Ed., pp. 250–3, Wiley, New York, 1963.
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