

Determination of DiazaCon in Quail Feed and Quail Serum by Ion Pair Reversed-Phase Chromatography

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Liquid chromatographic (LC) methods were developed for quantitating the potential avian contraceptive DiazaCon in quail feed and serum. DiazaCon was extracted from ground quail feed with basic *n*-butyl chloride. The *n*-butyl chloride extract was evaporated to dryness. The DiazaCon residues were dissolved in an aqueous methanolic ion pairing solution and quantitated by LC at 206 nm. Avian sera was combined with an equal volume of a pH 4 aqueous solution of ion pairing reagent and filtered to remove interfering proteins. DiazaCon was quantitated by LC. Mean recoveries for 500 and 2000 ppm fortified feed were 89.1 and 91.0%, respectively. The mean recovery for sera fortified at 5 levels ranging from 35 to 2000 ppm was 84.9%. Method limits of detection were approximately 14 and 13 ppm for feed and sera, respectively.

The U.S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Wildlife Services (WS) program is the federal program that provides leadership in managing problems that occur when human activity and wildlife are in conflict (1, 2). Human conflicts with avian species are numerous. Birds depredate food crops as well as present public health and aviation safety concerns. The National Wildlife Research Center (NWRC) devotes significant resources in developing nonlethal approaches to wildlife management. Cholesterol inhibition is one such nonlethal approach being investigated for control of pest bird populations. As cholesterol is a precursor for reproductive hormones, we are evaluating the effects of feeding the cholesterol analog DiazaCon (azacosterol, ornitrol, 20,25 diazacholesterol, diazasterol; Figure 1) on reproductive success in Coturnix quail (*Coturnix coturnix*). If DiazaCon reduces the levels of cholesterol available for hormone synthesis, reproductive success will likely diminish. The effects of DiazaCon administration on avian reproduction were previously investigated in red-wing blackbirds (3–6), pigeons (7, 8), house sparrows

(9, 10), grackles (6), and Japanese quail and parakeets (11). Unfortunately, the results of these studies are conflicting.

To increase the likelihood of success, *Coturnix* quail, a species that readily reproduces under laboratory conditions (12, 13), were used as the test species. Quail were fed a cholesterol free diet containing 0.1% DiazaCon for 14 days. With this design, the effects of 20,25 diazacholesterol inhibition on reproduction can be easily discerned. In previous studies, DiazaCon feed levels lower than 0.1% were found to be ineffective while higher levels produced mild toxic symptoms (14, 15). Efficacy (decreased laying) was noted after a minimum of 5 days of feeding, but feeding was not recommended for greater than 16 days (15). To better understand the mechanism(s) of efficacy, blood levels of DiazaCon, cholesterol, testosterone, and progesterone were monitored weekly.

Due to the lack of published analytical methodology, methods for the quantitation of DiazaCon in feed and sera needed to be developed. Our initial approach of adapting published methods for quantitating of cholesterol in blood (16–19) were unsuccessful, apparently due to the ionic nature of DiazaCon as compared to cholesterol. To permit chromatographic separation and subsequent quantitation of the DiazaCon, we formed an ion pair with heptanesulfonic acid and DiazaCon before analysis via an ion pair reversed-phase liquid chromatographic (LC) system.

METHOD

Reagents

(a) *Triethylamine*.—Reagent grade (Cat. No. 13,206-3; Aldrich Chemical Co., St. Louis, MO).

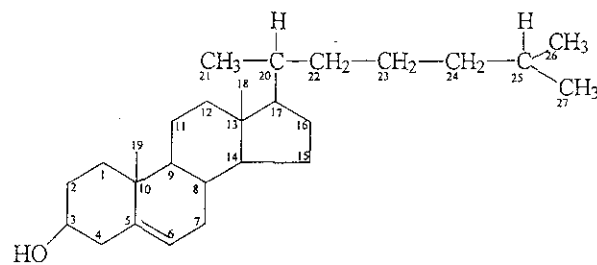
(b) *n*-Butyl chloride.—LC grade (Fisher Scientific, Fair Lawn, NJ) or equivalent.

(c) *Ion pairing reagent*.—Preformulated low UV heptanesulfonic acid IPC B7, Part No. 184282 (Alltech Associates, Inc., Deerfield, IL).

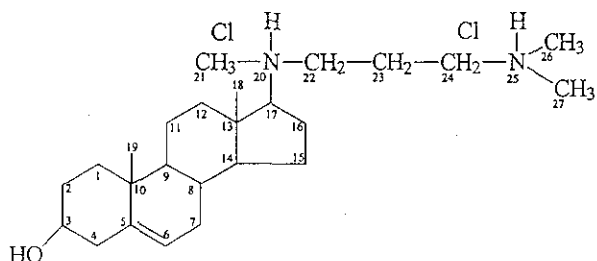
(d) *Methanol*.—LC grade (Fisher Scientific).

(e) *Water*.—Prepared with Milli-Q UV plus water purification system (Millipore Corp., Burlington, MA).

(f) *20,25 Diazacosterol [(3B,17B)-17-[[3 (dimethylamino)-propyl]methylamino]androst-5-en-3-ol]*.—99% pure (Avitrol Corp., Tulsa, OK). Dry at 110°C for at least 4 h and



Cholesterol



DiazaCon

Figure 1. Structures of cholesterol and DiazaCon.

then bring to ambient temperature by placing in a desiccator prior to use.

(g) *Phosphoric acid*.—Reagent grade, 4N (Fisher Scientific; 4N H₃PO₄). Dilute 9 mL concentrated phosphoric acid to 100 mL with H₂O.

(h) *Aqueous ion pairing solution*.—Prepare a 0.005M solution by mixing 20 mL ion pairing reagent with 1000 mL water. Adjust to pH 4.0 with 4N H₃PO₄ for sera.

(i) *Organic ion pairing solution*.—Prepare a 0.005M solution by mixing 20 mL ion pairing reagent with 1000 mL methanol (feed) or acetonitrile (sera).

(j) *Diluent*.—Mix 700 mL organic ion pairing solution with 300 mL aqueous ion pairing solution.

(k) *Base solution*.—Mix 100 mL triethylamine with 30 mL methanol and dilute with water to a final volume of 200 mL.

(l) *LC solution A*.—Mix aqueous and organic at (55 + 45; feed) or (70 + 30; serum) ion pairing solutions, and degas by sparging with helium.

(m) *LC column wash solution*.—Mix water and methanol (55 + 45; feed) or water and acetonitrile (50 + 45; serum). Degas by sparging with helium.

(n) *Chicken serum*.—Sigma (No. S-6773).

(o) *Quail feed*.—Game Bird Maintenance Chow, Purina (Gray Summit, MO).

Apparatus

(a) *Horizontal mixer/mechanical shaker*.—Eberbach Corp. (No. 6550; Ann Arbor, MI) or equivalent.

(b) *Ultrasonic bath*.—Branson (No. 5200; Garland, TX) or equivalent.

(c) *Glass test tubes*.—25 ± 150 mm (50 mL) with screw caps (No. 60828-285, VWR, Denver, CO) or equivalent.

(d) *Volumetric flasks*.—Pyrex 10 mL (5640-10), 25 mL (5640-25; VWR) or equivalent.

(e) *Eppendorf filtration tubes*.—Ultrafree PROBIND 45 µm filters (Millipore, Bedford, MA).

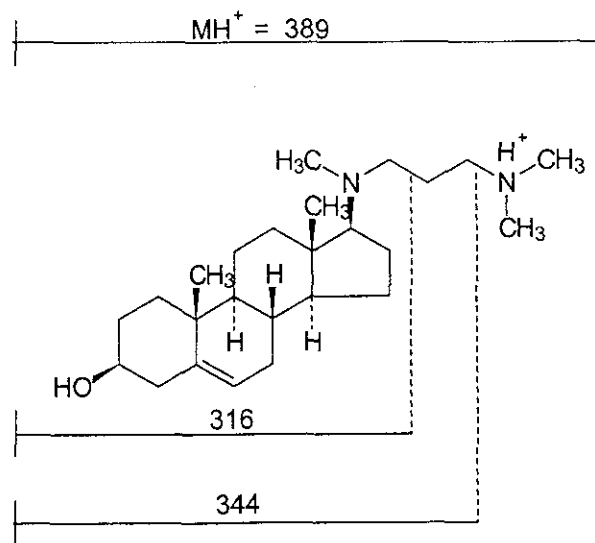


Figure 2. Mass spectral fragmentation pattern for DiazaCon.

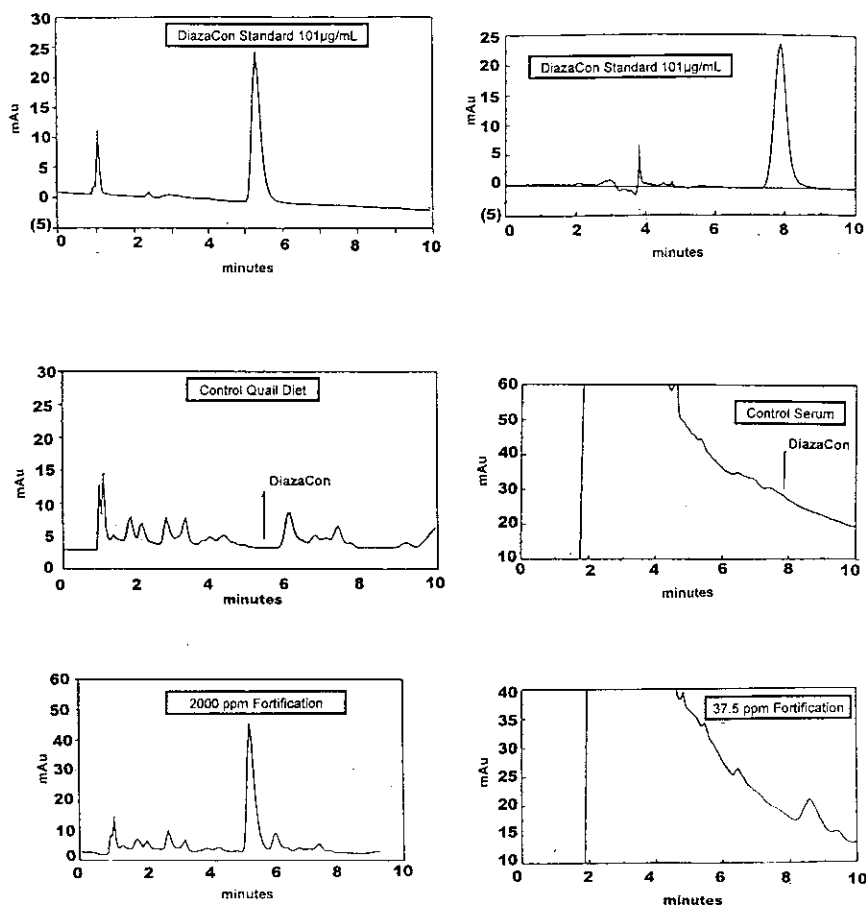


Figure 3. Liquid chromatograms of analytical standards, feed, and sera: 101 µg/mL analytical standard (top); control extract (middle); 2000 ppm fortified feed, and 37.5 ppm fortified serum (bottom).

(f) *Eppendorf standard (centrifuge) tube*.—1.5 mL (Brinkmann Instruments, Inc., Westbury, NY).

(g) *Microcentrifuge*.—Beckman Microcentrifuge E (Irvine, CA).

(h) *LC system*.—Hewlett Packard 1090 LC system (Palo Alto, CA) equipped with a diode array detector and operated at the following conditions: injection volume, 20 µL; column temperature, ambient (ca 25°C); UV absorbance, 206 nm; flow rate, 1 mL LC solution A/min (feed) or 0–8 min LC solution A at 0.4 mL/min, 8–12 min ramp to 90% organic ion pairing solution: 10% LC solution A at 0.6 mL/min (serum), 12–14 min hold, 14–16 min ramp to 100% LC solution A at 0.6 mL/min, 16–30 min 100% LC solution A at 0.4 mL/min (sera).

(i) *LC columns*.—(1) *Feed*.—Analytical column, 5 µm 100 × 4.6 mm id, C₁₈ Valupak LC column; guard column, (Keystone; Belefonte, PA) 5 µm, 12 × 4.6 mm id C₁₈ Valupak guard column (Keystone). (2) *Serum*.—Analytical column, 5 µm, 250 × 3 mm id ODS/H LC column (Keystone); guard column, 5 µm, 12 × 3 mm id ODS/H LC column (Keystone).

(j) *Mass spectrometer*.—LCQ ion trap mass spectrometer (Finnigan; San Jose, CA)

(k) *NMR spectrometer*.—Ace Spectrometer (Bruker, Inc. Billerica, MA) 300 MHz (¹H), 75 MHz (¹³C) 12 × 3 mm id.

(l) *pH meter*.—Beckman No. 45 pH meter and combination electrode or equivalent.

(m) *Blender*.—No. 1120 (Waring; Winstead, CT).

(n) *Vortex mixer*.—Touch Vortexer (Glas-Col; Terre Haute, IN).

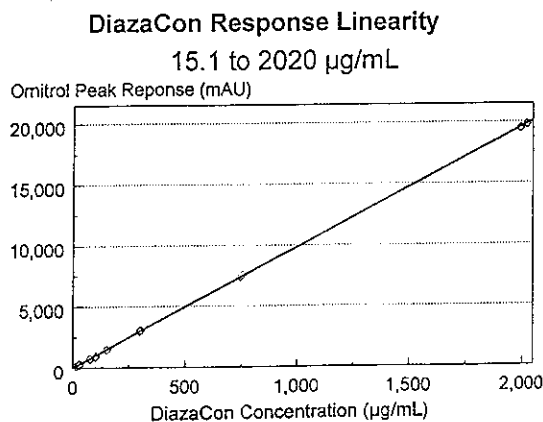


Figure 4. Response linearity for DiazCon.

Table 1. DiazaCon recoveries from sera and quail feed

Fortification level, ppm	<i>n</i>	Mean, % (SD)	CV, % (SD)
Sera			
35	7	69.3 (3.1)	4.5
150	7	78.5 (2.0)	2.5
600	7	93.9 (1.0)	1.1
1200	7	95.2 (2.4)	2.5
2000	7	87.5 (1.4)	1.6
Quail feed			
500	7	89.1 (3.0)	3.4
2000	7	91.0 (2.0)	2.2

Standard Solutions and Quality Control Samples

(a) *Concentrated standard solution.*—Accurately weigh ca 50 mg DiazaCon to the nearest 0.1 mg and quantitatively transfer to a 10 mL volumetric flask. Dilute to volume with methanol (feed) or water (sera) and mix to yield a concentrated stock solution of ca 5000 µg/mL.

(b) *Working standard solution.*—Transfer 200 µL concentrated standard solution to a 10 mL volumetric flask. Dilute to volume with diluent (feed) or aqueous ion pairing solution (sera). The concentration of the working standard solution is ca 100 µg/mL.

(c) *Linearity standard solutions.*—For the analysis of quail feed, prepare 2 sets of 5 calibration standards ranging from 25 to 250 µg/mL. Each set should be prepared from a separate independent stock solution. For the analysis of sera, standard concentrations should range from 15 to 2000 µg/mL.

(d) *Method validation samples.*—Fortify control sera at 0, 35, 150, 600, 1200, and 2000 µg/mL. Fortify quail feed at 500 and 2000 µg/g (0.05 and 0.2%) by adding a small quantity of water, blending to produce a mash (“cake batter” consistency), adding the required mass of Diaza Con (based on the dry weight of quail feed), followed by additional blending to produce a homogeneous feed.

(e) *Quality control samples.*—For the analysis of quail feed, fortify control quail feed at 0, 500, and 2000 µg/g. For the analysis of sera, fortify control sera at 0, 35, and 150 µg/mL.

Sample Preparation

(a) *Feed.*—Use a blender to grind the dried quail feed (ca 10 g) into a fine powder. Accurately weigh ca 2.5 g ground feed into a 50 mL glass test tube. Add 3.0 mL base solution, mix thoroughly on a Vortex mixer, and allow 5 min for the solution to saturate the feed. Add 20 mL *n*-butyl chloride to each tube, mix on Vortex mixer, cap, and shake on a horizontal mixer at high speed for 20 min. Centrifuge at ca 200 × *g* for 5 min. Transfer the supernatant to a clean glass test tube. Repeat extraction (and shaking) with *n*-butyl chloride and com-

bine butyl chloride supernatants. Evaporate to dryness under a gentle stream of nitrogen in a water bath at ca 60°C. Add ca 10 mL diluent, mix on Vortex mixer, and place in a sonicator for 20 min to ensure dissolution of the diazacholesterol residues. Mix again with a Vortex mixer. Quantitatively transfer the solution to a 25 mL volumetric flask with subsequent rinsing of the test tube with two 3–5 mL aliquots of diluent and transferring rinses to the volumetric flask. Fill the volumetric flask to volume with diluent. Using a syringe and 0.5 µm teflon filter, transfer 2 mL aliquots of extract to an amber LC autosampler vial and cap. Analyze by LC to quantitate DiazaCon.

(b) *Sera.*—Accurately transfer 50 µL avian serum to an Eppendorf centrifuge tube. Add 50 µL aqueous ion pairing solution (pH 4), cap, and mix on a Vortex mixer for 15 s. Transfer solution to an Eppendorf filtration tube, cap, and centrifuge at 500 × *g* for ca 4 min. Transfer the filtrate to an amber LC autosampler vial containing a 100 µL glass insert and analyze by LC to quantitate DiazaCon.

Sample Analysis

Prior to the LC analysis of sample extracts, confirm system suitability with consecutive injections of working standard solution to achieve a standard deviation for chromatographic area response ≤2%. To confirm response linearity, inject linearity standards in duplicate. Perform a linear regression analysis on the resulting mean chromatographic response vs concentration data to determine r^2 (coefficient of determination). To determine proportionality of chromatographic response, perform linear regression analysis on a log–log plot of these variables. To assess coextracted potential chromatographic interferences, analyze extracts of nonfortified control matrixes. Using the chromatograms from a control extract and the extract from a control feed or serum sample fortified at 500 µg/g or 40 µg/mL, respectively, calculate the method limit of detection (MLOD) as the concentration in a standard which would generate a response equivalent to 3 times the chromatographic response at the retention time of DiazaCon in the control chromatogram. At the end of the analysis day, columns are eluted with ca 60 mL column washing solution.

Chromatographic peak area is used to calculate the concentration of DiazaCon in sera as follows:

Table 2. DiazaCon recoveries from fortified quality control sera and feed samples

Fortification level, ppm	<i>n</i>	Mean, % (SD)	CV, %
Sera			
35	1	75.0	
150	3	80.2 (1.5)	2.1
Quail feed			
1000	9	80.4 (3.1)	3.9

$$\text{DiazaCon, } \mu\text{g/mL serum} = \frac{C_{\text{std}} (\mu\text{g/mL}) \times \frac{\text{Au}}{\text{As}} \times \frac{100 \mu\text{L}}{50 \mu\text{L serum volume}}}$$

For fortified quail feed, percent DiazaCon is calculated as follows:

$$C_{\text{std}} (\mu\text{g/mL}) \times \frac{\text{DiazaCon, \%}}{\text{As}} \times \frac{25\text{mL}}{\text{sample wt (g)}} \times \frac{1\text{g} \times 100}{1 \times 10^6 \mu\text{g}}$$

where Au = DiazaCon chromatographic response for sample; As = DiazaCon chromatographic response for working standard solution; and C_{std} = DiazaCon concentration in working standard solution.

Results and Discussion

As the DiazaCon analytical standard had been synthesized approximately 10 years prior to beginning the work reported here, purity was determined by elemental analysis, melting point analysis, mass spectrometry (MS), and nuclear magnetic resonance spectroscopy. Elemental analysis (CHN) was conducted by Desert Analytics (Tucson, AZ). Analytical results for percent C, H, and N of 77.3, 11.45, and 7.14%, respectively, were nearly identical to the theoretical values of 77.26, 11.41, and 7.20%. The observed melting point was 147°C (lit = 146°–148°C).

MS analysis was performed on a 1 $\mu\text{g}/\mu\text{L}$ solution of DiazaCon. To permit the identification of impurities, the solution was infused directly into the atmospheric pressure chemical ionization source without any preliminary chromatographic separations. MS analysis gave the expected MH^+ base ion of $m/z = 389$ (Figure 2). MS/MS analysis of the base ion resulted in $m/z = 344$, which is consistent with the loss of the positively charged dimethyl amine moiety. MS/MS/MS analysis of the 344 ion gave a base peak of $m/z = 316$, which is consistent with the loss of H_2CCH_2 .

Neither C-13 nor proton NMR completely resolved all 25 carbons or 46 hydrogens, respectively (Figure 1). However, all discernible data were consistent with the structure of DiazaCon. Carbon assignments were C-3, 72 ppm; C-5, 144 ppm; C-6, 123 ppm; C-17, 76 ppm; C-22, 56 ppm; C-23, 51 ppm; C-24, 55 ppm; C-26/27, 43 ppm; C-21, 42 ppm. Signals for the remaining 15 carbons were located between 41 and 10 ppm. Proton NMR results were H-3 (1H), 3.25 ppm; H-6 (1H), 5.2 ppm; H-17/22/24 (5H), 2.8 ppm; H-18/19 (6H), 0.9 ppm; H-20 (1H), 3.2 ppm. The hydroxyl H was undiscernible as it would be expected to be obscured by the solvent peak. Remaining hydrogens were located between 2.2 and 0.9 ppm. These locations are consistent with the expected results. Peak assignments versus noise permitted a maximum of 10% undetected impurities in the carbon spectra and 1–2% in the proton spectra. The NMR analyses indicate the presence of no impurities greater than 1–2% in the technical standard. Analysis of the standard by elemental analysis, melting point

analysis, MS, and NMR fail to indicate the presence of any impurities in the DiazaCon analytical standard.

Chromatograms of fortified and blank sera and quail feed are shown in Figure 3. Due to the consistent slope of the baseline, integration of diazacholesterol peak area was easily accomplished. Under the specified conditions, the retention time for diazacholesterol is approximately 7.5 min for quail feed extracts and 8.0 min for sera extracts. The MLOD for the sera, based on the chromatographic response from 40 ppm fortified sample, was 13 ppm. The MLOD for feed, based on the chromatographic response from a 500 ppm fortified sample was approximately 14 ppm. Response linearity (chromatographic response versus concentration) for DiazaCon in the LC system was excellent, as r^2 was >0.995 (Figure 4). For the log–log plots, slopes ranged from 0.998 to 1.02 and were not significantly different from 1.0, indicating a proportional response.

Data for recoveries of DiazaCon from both sera and quail feed fortified at several levels are shown in Table 1. Recoveries from sera ranged from 69.3% at 35 ppm to 95.2% at 1200 ppm. The fortification range was more narrow for quail feed than sera as the feed was prepared at a target concentration of 1000 ppm (0.10%). Feed was fortified at 50 and 150% of this target concentration. Mean recoveries ranged from 89.1 to 91.0%. For both matrixes, variation decreased with increasing fortification levels. However, variation was acceptable at all fortification levels as the coefficient of variation (CV) never exceeded 4.5%.

This method was used to confirm the concentration of DiazaCon in 39 quail feed and 60 quail sera samples. With each set of 10 samples, a blank and fortified quality control sample were also analyzed. No chromatographic interferences were noted in any of the blank extracts. Recoveries for the fortified QC samples analyzed concurrently with study samples are presented in Table 2. For sera, the mean recovery for the 150 ppm QC samples was 80.2%, while the recovery of the 35 ppm QC sample was 75.0%. The mean recovery of the 1000 ppm feed samples was nearly identical at 80.4%. While QC recoveries for both matrixes were similar, the variability for the sera samples was less than half that observed for the quail feed.

The corrected mean DiazaCon concentration in the medicated feed was 1085 ppm. While this mean concentration was within 10% of the target concentration, the CV was 29%. This suggests that the medicated feeds should be prepared more carefully and/or the formulation procedure could be improved. As the baits were prepared under field conditions, the precision could likely be improved by preparing the baits in an analytical or formulation lab. The medicated feed then could be subsequently transported to the required site. No DiazaCon residues were detected in the sera samples in spite of the fact that the dose was efficacious (number of eggs laid decreased by 75 to 90%). The sera residue data suggest that the blood does not harbor significant residues of DiazaCon. This method proved sufficiently rugged to confirm the concentration of DiazaCon in medicated feed and to establish that DiazaCon has excellent potential as a contraceptive for nonlethal control of pest bird populations.

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