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Determination of 4,4′-Dinitrocarbanilide (DNC), the Active Component of the Antifertility Agent Nicarbazin, in Chicken, Duck, and Goose Plasma

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4,4′-Dinitrocarbanilide (DNC) was extracted from chicken, duck, and goose plasma and isolated by reversed-phase high-performance liquid chromatography. DNC was detected by ultraviolet absorbance at 347 nm and quantified by comparison to a calibration standard. Recovery data were determined by analyzing DNC-fortified control plasma. The mean recovery of DNC in fortified chicken plasma samples was 99.7 ± 1.9% for 0.18 and 9.1 ppm DNC, and in fortified duck and goose plasma samples was 99.5 ± 4.9% and 101.4 ± 4.5%, respectively, for 0.18, 9.1, and 18 ppm DNC.

Keywords: *4,4′-Dinitrocarbanilide; nicarbazin; high-performance liquid chromatography; plasma; chicken; duck; goose*

INTRODUCTION

Canada geese are commonly thought of as migrating birds. However, the number of nonmigrating (resident) Canada geese is increasing (*1*). Generally, people usually accept a few Canada geese as pleasant. However, as the number of resident geese increases, problems such as the fouling of water supplies, lawns, beaches, and golf courses with excreta, overgrazing of grassy areas, and flocks feeding on crops such as corn, soybeans, rice, lettuce, and wheat occur more frequently (*2*). Recommended management techniques for Canada geese and their associated problems include use of scaring devices, dogs to chase geese, prevention of nesting, installation of barriers, reducing feeding practices by the public, adjusting landscaping practices, relocating birds, and utilizing hunting practices (*3*). Reducing Canada geese populations in resident flocks would help to alleviate many of the problems associated with this species. Thus, an antifertility agent used on a limited basis for a growing pest species such as the Canada goose may reduce numbers to a desirable and manageable level.

Nicarbazin is an FDA-approved drug used for the treatment and prevention of coccidiosis in broiler chickens. Nicarbazin is an equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 4,6-dimethyl-2-pyrimidinol (HDP). When nicarbazin was accidentally fed to breeder chickens, decreased egg hatchability was observed. The active component of nicarbazin, DNC, is responsible for the decreased hatchability of eggs. The National Wildlife Research Center is evaluating nicarbazin as a potential antifertility agent for Canada geese. It is hoped that correlations between nicarbazin diet concentration, nicarbazin dose, blood DNC levels, and hatchability will permit the determination of efficacious, yet safe, Nicarbazin diet concentrations for multiple avian species. To help bridge our future findings with the extensive Nicarbazin database for chickens, chickens as well as Canada geese and mallard ducks were used as test species. To achieve our research goals, analytical methods needed to be developed for the quantification of DNC in plasma.

Initially chickens, mallards, and Canada geese were dosed with nicarbazin at 8.4 mg/kg and plasma samples were collected, frozen, and analyzed. Plasma data for chicken have been reported for DNC and HDP but no method was published (currently unpublished proprietary data). The Analytical Chemistry Project (ACP) at the National Wildlife Research Center (NWRC) developed and validated a method for the determination of DNC in the plasma of chickens, Canada geese, and mallards.

Methods for the analysis of nicarbazin typically focus on the residue determination of DNC in eggs and chicken muscle tissue. The HDP component of the complex increases the adsorption of DNC into the circulatory system. The HDP that is adsorbed into the blood stream is excreted at a much faster rate than DNC (*4*, *5*). Most residue methods for DNC include a sample cleanup. Most cleanup steps are accomplished by liquid−liquid extraction, solid-phase extraction columns, or online columns prior to the analytical column in the HPLC analysis (*6*−*9*). Others have used liquid chromatography/mass spectrometry (LC/MS) to avoid sample extract cleanup (*10*−*12*). Unfortunately, LC/MS is not a widely available technique. To accomplish our research goals, we developed a simple HPLC method for the quantification of DNC in avian plasma. This method uses very small solvent volumes for the extraction with high sample throughput. The resulting data will be used to determine target Nicarbazin dose levels for field studies to develop nicarbazin as an infertility agent in Canada

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Table 1. HPLC Parameters

mobile phase	60% acetonitrile 40% water
flow rate	1.0 mL/min
oventemperature	35 °C
column	Keystone ODS/H, 25.0 cm × 4.6 mm i.d., 5 μm or equivalent with 1.5 × 4.6 mm i.d. guard column
injection volume	60 μL
detector	UV @ 347 nm
run time	11 min (approximate retention time of the analyte under the above conditions is 8.5 min)

geese. Data from controlled mallard studies combined with Canada goose field trials will eventually be provided as data submissions to the U. S. Food and Drug Administration.

MATERIALS AND METHODS

Reagents. Acetonitrile (Fisher Scientific, Denver, CO) was liquid chromatography grade. *N*,*N*-Dimethylformamide (Fisher Scientific, Denver, CO) was reagent grade. Deionized water was purified using a system combining ion-exchange resin cartridges and UV irradiation to produce 18 mega-ohm water.

Standard Preparation and Calibration Curve. DNC with a purity of 99.0% was obtained from Chem Service (West Chester, PA). Concentrated fortification stock solutions of 100 ppm DNC were prepared from the commercial products by dissolving 5 mg in 50 mL of dimethylformamide (DMF). Working solutions were prepared every week by dilution with mobile phase. All standard solutions were stored in the dark at 22−24 °C.

For plasma analysis, five DNC working solutions (0.050 μg/mL to 8 μg/mL) were prepared and analyzed by HPLC in duplicate. A plot was constructed of DNC chromatographic peak response (*y*-axis) vs DNC concentration (*x*-axis). Linear regressions were performed on the data (SAS, Cary, NC).

Sample Preparation. A 100-μL aliquot of each plasma sample was transferred into a 1.5-mL plastic Eppendorf microcentrifuge tube. The samples were diluted with 200 μL of acetonitrile and vortex mixed. The samples were centrifuged in an ultracentrifuge (Beckman Microfuge E) for 5 min. The supernatant was accurately transferred into a 350-μL glass insert (Supelco, Bellafonte, PA), which had been placed into a HPLC vial and capped. An injection of 60 μL was completed for each sample and quality control sample into the HPLC and the concentration of DNC was determined versus calibration standards.

Fortification of Control Plasma. A DNC concentrated standard solution (1000 μg/mL) was prepared by accurately weighing 50 mg of DNC reference standard into a 50-mL volumetric flask. The DNC was dissolved and diluted to volume with DMF. Fortification standard solutions were prepared by dilution of the concentrated standard solution to 200, 100, and 2 μg/mL in DMF in 10-mL volumetric flasks. For 0.18 μg/mL fortified samples, 10.0 μL of the 2.0 μg/mL standard solution was added to 0.100 mL of control plasma and vortex mixed. Likewise, for samples fortified at the 9.1 or 18 μg/mL, 10.0 μL of the 100 or 200 μg/mL standard solution was added to 0.100 mL of control plasma and vortex mixed. We then proceeded with the extraction procedure as described above.

Chromatographic System. Samples were analyzed by a Hewlett-Packard 1090M high-performance liquid chromatograph (HPLC) equipped with a Hewlett-Packard diode array ultraviolet−visible detector. The HPLC parameters utilized are listed in Table 1. The DNC chromatographic response was identified by comparison with the retention time and UV−visible spectra of a standard and quantified with the use of external calibration standards. A Hewlett-Packard computer work station with chromatographic software and printer were used to collect, process, store, and print the chromatographic data.

Table 2. Recovery Data from DNC-Fortified Plasma

Chicken

sample no.	0.18 ppm DNC	9.1 ppm DNC
1	96.9	99.2
2	94.9	99.6
3	94.9	99.0
4	96.9	99.2
5	96.4	99.5
6	97.4	99.0
7	94.4	99.9
mean	96.0%	99.3%
s =	1.2%	0.34%
cv =	1.2%	0.34%

Mallard

sample no.	0.18 ppm DNC	9.1 ppm DNC	18 ppm DNC
1	81.6	98.2	103
2	96.6	96.9	101
3	99.4	96.6	102
4	102	98.9	104
5	104	97.7	104
6	103	98.2	102
7	103	97.2	99.4
mean	98.5%	97.7%	102%
s =	7.9%	0.82%	1.7%
cv =	8.0%	0.84%	1.7%

Canada Goose

sample no.	0.18 ppm DNC	9.1 ppm DNC	18 ppm DNC
1	103	96.0	105
2	97.2	97.5	106
3	102	96.6	104
4	106	95.9	92.2
5	107	96.8	103
6	104		106
7	104		104
mean	103%	96.6%	103%
s =	3.2%	0.65%	4.8%
cv =	3.1%	0.67%	4.7%

RESULTS AND DISCUSSION

Response Linearity. Two sets of six DNC standard solutions were prepared ranging from 0.050 to 8 μg/mL. Data were collected from duplicate injections of each solution, and a plot was constructed of analyte peak response (*y*-axis) vs DNC concentration (*x*-axis). A linear regression was performed on the data set and produced a $r^2 = 0.9999$. The plot of log(analyte response) vs log(DNC concentration) produced a slope = 1.025917 and a $r^2 = 0.9998$. A linear and proportional relationship exists between chromatographic peak response and DNC concentration from 0.05 μg/mL to 8.0 μg/mL. Single point calibration is valid in this range.

Matrix Interference. Six control plasma samples for each avian species were analyzed according to the procedures described. No chromatographic interferences were observed at or near the retention time of DNC. Chromatograms of control chicken, duck, and goose plasma samples were virtually identical. Therefore, only chromatograms of a control and fortified (0.18 ppm) goose plasma are shown in Figure 1A and 1B.

Instrument Limit of Detection (ILOD). The instrument limit of detection (ILOD) was estimated from the mean chromatographic response of three reagent blanks and the response of a 0.0515-μg/mL DNC standard. The ILOD was defined as the concentration of DNC required to generate a signal equal to 3× the baseline noise (measured peak-to-peak) observed in the reagent blank. Under the conditions stipulated in the method, the ILOD for DNC is equal to 0.011 μg/mL.

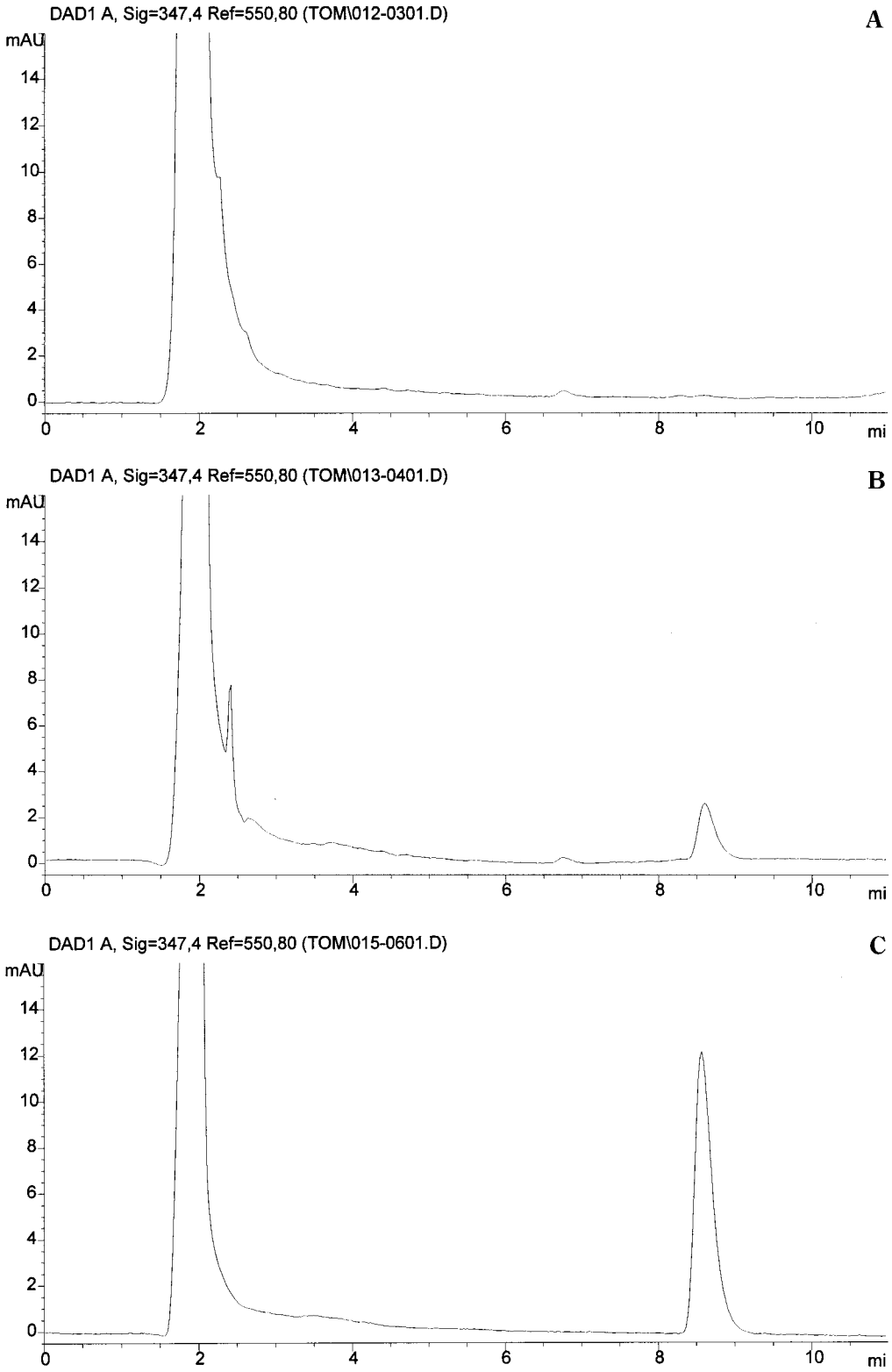

Figure 1. Chromatograms of a control goose plasma sample (A), a goose plasma sample fortified at 0.18 ppm (B), and a goose plasma sample from a bird treated with 8.4 mg/kg nicarbazin (C).

Method Limit of Detection (MLOD). The method limit of detection (MLOD) was estimated from the mean chromatographic response of three control plasma samples for each type of plasma and the response of a control plasma fortified at 0.18 μg/mL DNC (at least four replicates were fortified for each type of plasma). The MLOD was defined as the concentration of DNC required to generate a signal equal to 3× the baseline noise (measured peak-to-peak) observed in the control sample. The MLODs for DNC in chicken, duck, and goose plasma were 0.033, 0.027, and 0.035 μg/mL, respectively.

Bias and Repeatability. Replicate control plasma samples were fortified with DNC and assayed according to the procedures in this method. The mean recoveries of DNC from chicken, duck, and goose plasma were 97.7 ± 1.9%, 99.5 ± 4.9%, and 101.4 ± 4.5%, respectively. The recovery and precision data are shown in Table 2.

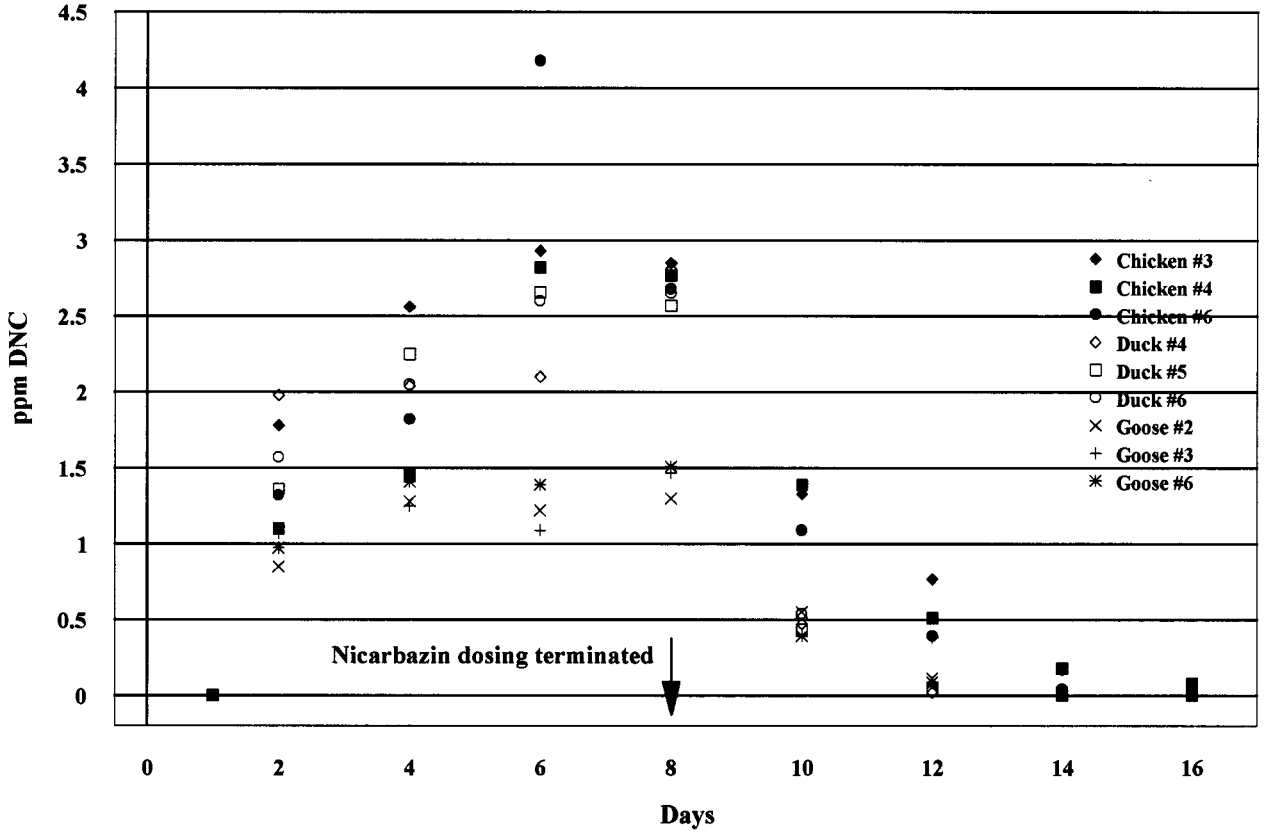

Figure 2. DNC concentration in plasma from individual chickens, mallard ducks, and Canada geese dosed with Nicarbazin.

Method Performance. The usefulness of the method was demonstrated by the analyses of chicken, duck, and goose plasma collected during a study to assess the DNC plasma levels of birds dosed with Nicarbazin at 8.4 mg/kg for 8 days followed by 8 days of no treatment. Plasma samples were drawn every other day over the duration of the study. Analysis of 230 plasma samples required 11 working days. The mean recoveries of DNC fortified (0.18 and 9.1 μg/mL) chicken, duck, and goose plasma were 97.5 ± 3.7%, 97.7 ± 5.7%, and 101.4 ± 6.7%, respectively, for this study. A portion of the data is presented in Figure 2. The data points represent the average DNC concentration in the plasma for three individual birds in each test group. For all 3 species, DNC plasma levels reached a plateau after 6 days of treatment. DNC plasma levels rapidly decreased following cessation of nicarbazin treatment. Within 4 days, duck and goose DNC plasma levels decreased to less than the MLOD, whereas chicken required 6 to 8 days to reach this level. A goose plasma sample collected on day 6 from a bird treated with 8.4 mg/kg and assayed with the method is shown in Figure 1C.

CONCLUSION

This methodology developed for the determination of DNC in avian plasma proved to be reliable, efficient, and simple, with high sample throughput. The same method was used for plasma from three different species. The mean recovery of DNC in fortified avian plasma samples was 99.6 ± 4.3% for 0.18, 9.1, and 18 ppm DNC.

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