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DRUGS, COSMETICS, FORENSIC SCIENCES

Liquid Chromatographic Determination of 4,4′-Dinitrocarbanilide, the Active Component of the Infertility Agent Nicarbazin, in Chicken, Duck, Goose, and Snake Eggs

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4,4′-Dinitrocarbanilide (DNC) was extracted from chicken, duck, goose, and snake eggs and isolated by reversed-phase liquid chromatography. DNC was detected by ultraviolet absorbance at 347 nm and quantitated by comparison with a calibration standard. Recoveries of DNC from fortified control chicken, duck, goose, and snake egg samples were determined for DNC levels of 0.16, 10, and 16 μg/g. The mean recoveries from chicken, duck, goose, and snake eggs were 92 ± 4, 88 ± 9, 87 ± 7, and 95 ± 6%, respectively. The method limits of detection for DNC in chicken, duck, goose, and snake eggs ranged from 0.015 to 0.035 μg/g. The reported method is much simpler than and equally efficient as previous methods developed for the determination of DNC residues in egg contents.

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. As the number of resident geese increases, problems such as the following occur more frequently (2): fouling of water supplies, lawns, beaches, and golf courses with excreta; overgrazing of grassy areas, and feeding on crops such as corn, soybeans, rice, lettuce, and wheat by flocks. Recommended management techniques for Canada geese and their associated problems include using scaring devices and dogs to chase geese, preventing nesting, installing barriers, reducing feeding practices by the public, adjusting landscaping practices, relocating birds, and utilizing hunting practices (3). Reducing populations of Canada goose in resident flocks would help to alleviate many of the problems associated with this species. Thus, an infertility agent used on a limited basis for a pest species such as Canada geese may reduce numbers to a desirable and manageable level.

Nicarbazin is a drug approved by the U.S. Food and Drug Administration (FDA) for the treatment and prevention of coccidiosis in broiler chickens. Nicarbazin (Figure 1) is an equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 4,6-dimethyl-2-pyrimidinol (HDP). It was discovered in the mid-1950s when nicarbazin was accidentally fed to breeder chickens and decreased egg hatchability was observed (4). The active component of nicarbazin, DNC, was responsible for the decreased hatchability. The National Wildlife Research Center (NWRC) is evaluating nicarbazin as a potential infertility agent for Canada geese. The goal is to evaluate the correlations between nicarbazin diet concentration, nicarbazin dose, plasma and egg DNC levels, and hatchability that 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 mallards were used as test species. To achieve our research goals, analytical methods were needed for the determination of DNC in plasma (5) and eggs. The Analytical Chemistry Project at the NWRC developed and validated a method for the determination of DNC in the eggs of chickens, Canada geese, and mallards.

Methods for nicarbazin analysis typically focus on the determination of DNC residues in chicken eggs and muscle tissue. The HDP component of the complex increases the adsorption of DNC into the circulatory system. The HDP that is adsorbed into the bloodstream is excreted at a much faster rate than is DNC (6, 7). Most residue methods for DNC include a sample cleanup. Most cleanup steps use liquid–liquid extraction, solid-phase extraction columns, or on-line columns before liquid chromatography (LC; 8–11). Others have used LC/mass spectrometry (LC/MS) to avoid cleanup of the sample extract (12, 13). Unfortunately, LC/MS is not a widely available technique.

To accomplish our research goals, we developed a simple LC method for the determination of DNC in avian eggs. This method is simple and efficient, allowing for high sample throughput. The resulting data from the application of this methodology will be used to determine target nicarbazin dose levels for field studies to develop nicarbazin as an infertility

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agent in Canada geese. Data from controlled mallard studies combined with Canada goose field trials will eventually be provided as data submissions to the FDA.

Development of infertility agents for invasive species, such as brown tree snakes, that cause damage to endangered species is the goal of some biologists at the NWRC. Methods to determine the levels of DNC in snake plasma and eggs are required to help assess the efficacy of nicarbazin to reduce hatchability in snakes. Because brown tree snakes do not breed well in captivity, the African house snake is being tested as the model species. The method developed to determine DNC residues in avian eggs was applied to eggs produced by African house snakes exposed to nicarbazin.

Experimental

Apparatus

Samples were analyzed by using a Hewlett-Packard (Palo Alto, CA) 1090M liquid chromatograph equipped with a Hewlett-Packard diode array UV-Vis detector. The LC parameters used are listed in Table 1. The DNC chromatographic response was identified by comparison with the retention time and UV-Vis spectrum of a standard and quantitated by the use of external calibration standards. A Hewlett-Packard computer workstation with chromatographic software and a printer were used to collect, process, store, and print the chromatographic data.

Reagents

(**a**) *Acetonitrile (ACN).*—LC grade; Fisher Scientific (Denver, CO).

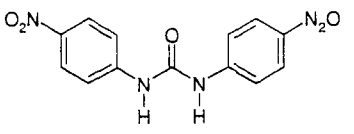
(**b**) *N,N-dimethylformamide (DMF).*—Reagent grade (Fisher Scientific).

(**c**) *Deionized water.*—Purified by using a system combining ion-exchange resin cartridges and UV irradiation to produce 18 megaohm water.

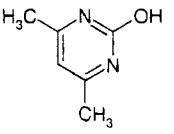
Preparation of Standard Solutions and the Calibration Curve

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

During method validation of egg analysis, DNC working solutions (0.025–5 μg/mL) were prepared and analyzed in duplicate by LC. A plot was constructed of DNC chromatographic peak response (y-axis) vs DNC concentration (x-axis). Linear regressions were performed on the data by using SAS software (Cary, NC).



DNC



HDP

Figure 1. Structure of nicarbazin, an equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 4,6-dimethyl-2-pyrimidinol (HDP).

Table 1. LC parameters for the analysis of chicken, duck, goose, and snake eggs

Parameter	Chicken, duck, and goose	Snake
Mobile phase	ACN–water (60 + 40)	ACN–water (55 + 45)
Flow rate, mL/min	1.0	
Oven temp, °C	35	
	Keystone ODS/H, 25.0 cm × 4.6 mm id, 5 μm, or equivalent, with 1.5 × 4.6 mm id guard column	
Injection volume, μL	60	
Detector	UV at 347 nm	
Run time, min	15[a]	

[a] Approximate retention time of the analyte under the above conditions is 8.8 min for chicken, duck, and goose samples and 11.7 min for snake samples.

Sample Preparation

A 5 g portion of homogenized egg, including the shell, was transferred to a 25 mL glass tube and weighed. The sample was extracted with 7 mL ACN–DMF (1 + 1), mixed on a Vortex mixer, and placed in an ultrasonic bath for 10 min. The samples were centrifuged for 5 min at 1000 × g, and the supernatant was transferred to a 25 mL volumetric flask. The extraction was repeated 2 more times with 7 mL portions of ACN–DMF (1 + 1). The supernatant was diluted with deionized water to 25.00 mL, mixed well, and filtered through a 0.45 μm Teflon syringe filter into an LC vial, and the vial was capped. The LC injection volume was 60 μL for each sample extract and standard solution.

Fortification of Control Egg

All control eggs used for quality control samples were collected from control animals before initiation of the studies. A concentrated standard solution of DNC (1000 μg/mL) was prepared by accurately weighing 50 mg DNC reference standard into a 50 mL volumetric flask. The DNC was dissolved in DMF, and the solution was diluted to volume with DMF. A second fortification standard solution was prepared by dilution of the concentrated standard solution to 10.0 μg/mL in DMF in a 10 mL volumetric flask. For samples fortified at 0.16 μg/g, 80.0 μL standard solution at 10.0 μg/mL was added to 5.00 g control egg and mixed on a Vortex mixer. Likewise

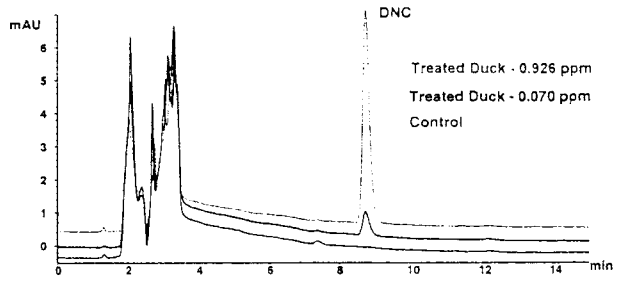


Figure 2. Chromatograms of duck egg extracts from 3 different treatment groups. For the avian egg analysis, the mobile phase consisted of ACN–water (60 + 40).

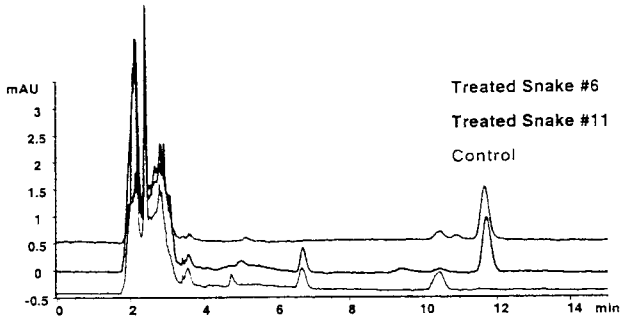


Figure 3. Chromatograms of extracts of eggs from 2 snakes dosed with nicarbazin and a control snake. For the snake egg analysis, the mobile phase consisted of ACN–water (55 + 45).

for samples fortified at 10 or 16 μg/g, respectively, 50.0 or 80.0 μL standard solution at 1000 μg/mL was added to 5.00 g control egg and mixed on a Vortex mixer. Fortified egg matrixes were then extracted as described above.

Results and Discussion

Linearity of Response

Two sets of 6 DNC standard solutions were prepared, ranging from 0.025 to 5 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 performed on the data set produced a value of $r^2 = 0.9999$. The plot of log (analyte response) vs log (DNC concentration) produced a slope = 1.025917 and $r^2 = 0.9998$. A linear and proportional relationship existed between chromatographic peak response and DNC concentration from 0.05 to 5.0 μg/mL. Single-point calibration was valid in this range.

Matrix Interference

Seven control egg samples from a composite of 7 eggs 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 and treated chicken, duck, and goose egg samples were virtually identical and, therefore, only chromatograms of extracts of mallard duck eggs are shown in Figure 2. Because of the limited number of snake eggs and their smaller mass (3–5 g), individual control eggs were used for quality control samples and not composites. Chromatograms of extracts of an African house snake control egg and of eggs from 2 snakes dosed with a nicarbazin-fortified diet are shown in Figure 3.

Instrument Limit of Detection (ILOD)

The ILOD was estimated from the mean chromatographic response for 3 reagent blanks and the response for a DNC standard at 0.025 μg/mL. The ILOD was defined as the con-

Table 2. Mean recovery (%) of DNC from fortified chicken, duck, goose, and snake eggs

	Fortification level, μg/g				
Species	0.16	10	16	All levels	Total replicates analyzed
Chicken	91 ± 4.9	91 ± 3.7	93 ± 1.7	92 ± 4.2	93
Duck	90 ± 12	—	88 ± 3.1	88 ± 8.6	72
Goose	89 ± 7.5	84 ± 5.7	89 ± 1.2	87 ± 7.1	175
African house snake	94 ± 9.2	95 ± 3.2	—	95 ± 5.6	12

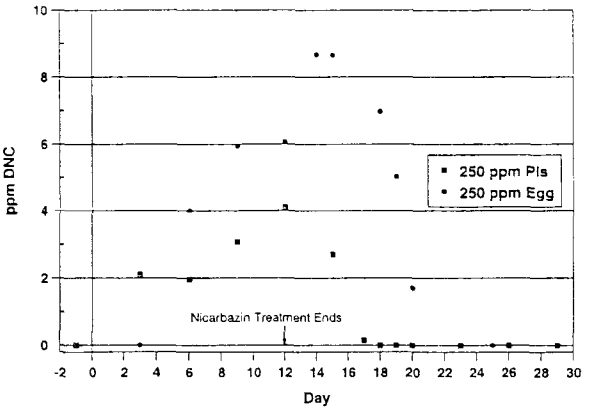


Figure 4. Concentration of DNC in plasma versus eggs from a duck fed a diet containing 250 ppm nicarbazin for 12 days followed by 12 days of control diet.

centration of DNC required to generate a signal equal to 3× the baseline noise (measured peak to peak) observed for the reagent blank. Under the conditions stipulated in the method, the ILOD values for DNC were 0.007 μg/mL for the avian egg method and 0.009 μg/mL for the snake egg method.

Method Limit of Detection (MLOD)

The MLOD was estimated from the mean chromatographic response for 3 control eggs for each type of egg and the response for a control egg fortified with DNC at 0.16 μg/g. For validation, 7 replicates were fortified for each avian egg type, and 4 replicates for the African house snake egg. 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 for the control sample. The MLOD values for DNC in chicken, duck, goose, and snake eggs were 0.033, 0.027, 0.035, and 0.015 μg/g, respectively.

Bias and Repeatability

Replicate control egg samples were fortified with DNC and analyzed according to the procedures in this method. The mean recovery for each fortification level is shown in Table 2 for each species. The number of values used for each mean is a reflection of the number of samples analyzed over the course of multiple studies. All of these means are therefore representative of analyses over multiple days and typically multiple analysts. The mean recoveries of DNC for all levels from chicken, duck, goose, and snake eggs were 92 ± 4, 88 ± 9, 87 ± 7, and 95 ± 6%, respectively. Initially, the method was validated for fortification levels of 0.16 and 10.0 μg/g, although when chicken and goose egg samples from studies were analyzed, it was necessary to increase the high level to 16.0 μg/g. Therefore, the 10.0 μg/g fortification level for duck eggs was eliminated. Snake eggs did not require a fortification level of 16.0 μg/g.

Method Performance

The usefulness of the method was demonstrated by the analyses of chicken, duck, and goose eggs collected during a study to assess the DNC levels in plasma (5) and eggs of birds fed a diet containing 4 different levels of nicarbazin for 12 days followed by 12 days of no treatment. Plasma samples were drawn on the days shown in Figure 4, and all the eggs produced were collected over the duration of the study for each test animal. An example of the usefulness of the egg and plasma method is presented in Figure 4 for one of the study animals. The data points represent the average DNC concentration in the eggs and plasma of an individual mallard in the 250 ppm nicarbazin test group. DNC plasma levels rapidly decreased after the cessation of nicarbazin treatment. Within 4–6 days, DNC plasma levels decreased to less than the MLOD, whereas the DNC egg levels were less than the MLOD after approximately 11–13 days. To date, the method has been used to analyze >2100 samples, 110 control samples, and 340 fortified samples.

Conclusions

The methodology developed for the determination of DNC in avian eggs proved to be reliable, efficient, and simple, with high sample throughput. The same method was used for eggs from 3 different avian species, with a mobile phase modification used for snake eggs. The mean recoveries of DNC from chicken, duck, goose, and snake eggs were 92 ± 4, 88 ± 9, 87 ± 7, and 95 ± 6%, respectively. The overall mean recovery of DNC from fortified egg samples was 90 ± 4% for 0.16, 10, and 16 ppm DNC, with a mean MLOD of 0.028 μg/g.

Acknowledgments

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