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Desmosterol: A Biomarker for the Efficient Development of 20,25-Diazacholesterol as a Contraceptive for Pest Wildlife

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20,25-Diazacholesterol is being evaluated as a contraceptive for the nonlethal control of avian and mammalian wildlife pests. The identification of an analyte in blood which was highly correlated with absorbed dose and efficacy is valuable for determining effective formulations and dosing variables. Such an analyte or biomarker is also valuable for determining the percentage of pest populations that consume an effective dose of the active ingredient in the field. HPLC analyses of blood collected from dosed animals failed to detect 20,25-diazacholesterol but indicated that levels of free cholesterol and related compounds were affected by 20,25-diazacholesterol absorption. The greatest percent change in chromatographic peak area associated with 20,25-diazacholesterol administration was observed for desmosterol, a cholesterol precursor. 20,25-Diazacholesterol appeared to block the conversion of desmosterol to cholesterol, resulting in an elevated concentration of the precursor. The elevation of blood desmosterol levels is being used as an indicator of 20,25-diazacholesterol absorption and to facilitate the development of a 20,25-diazacholesterol-based contraceptive for pest wildlife.

KEYWORDS: Wildlife; contraception; cholesterol; HPLC; biomarker

INTRODUCTION

Human conflicts with wildlife are numerous. Birds and mammals depredate food crops as well as present public health and aviation safety concerns (*1*). The National Wildlife Research Center (NWRC) devotes significant resources to developing nonlethal approaches for wildlife management. Cholesterol inhibition is one such nonlethal approach being investigated for control of pest wildlife populations. As cholesterol is a precursor for reproductive hormones, we hypothesized that suppression of cholesterol levels in pest wildlife will have a contraceptive effect. For many herbivorous animals such as deer and grainivorous birds, all of the cholesterol is synthesized within the body. It is for these animals that we believe this approach to population control is most promising.

One compound being evaluated as a wildlife contraceptive is the cholesterol analogue 20,25-diazacholesterol (**Figure 1**) also known as azacosterol, ornitrol, diazacon, and diazasterol. In the early 1960s G.D. Searle Inc. studied the hypocholesterolemic activity of nitrogen-substituted analogues of cholesterol as a potential treatment for heart disease involving cholesterol deposits in the coronary arteries. Of the various compounds studied, 20,25-diazacholesterol was noted to be an extremely potent inhibitor of cholesterol synthesis in laboratory animals (*2*). It was suggested that the hypocholesterolemic effect of 20,25-diazacholesterol is likely due to the interaction of the cationic amino substituent at the 25 position and the complimentary anionic site on the HMG-CoA enzyme receptor. The

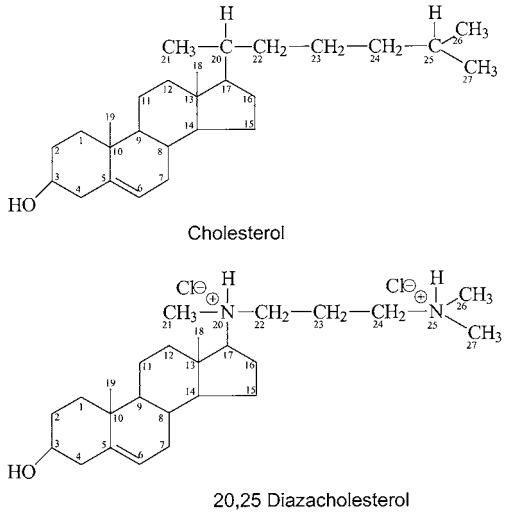

Figure 1. Cholesterol and 20,25-diazacholesterol.

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net result is that 20,25-diazacholesterol has a higher affinity than cholesterol for the enzyme receptor. Because of this high affinity for the rate-limiting enzyme in the cholesterol synthesis sequence, 20,25-diazacholesterol has a negative feedback effect on cholesterol synthesis at a low dose (*3*). Additionally, it has been reported that the amino substituent at the 20 position competes with cholesterol for the receptor site on the cytochrome P450 enzyme which is responsible for the cleavage of the cholesterol side chain. This competitive binding to the enzyme reduces the opportunity for cholesterol to be cleaved and therefore reduces the synthesis of pregnenolone and subsequent sex hormones (*4*). By these two modes of action, it is believed that 20,25-diazacholesterol reduces both the overall levels of cholesterol in the body and the cholesterol side chain cleavage needed for the production of the reproductive hormones.

To develop 20,25-diazacholesterol into an effective tool for the control of pest wildlife populations, effective dosing procedures and formulations must be developed. Unfortunately, development of such procedures and formulations is stymied by a single annual breeding period, long gestation time, and small litter size of many pest wildlife species. For example, white-tail deer have a gestation time of 200 days and typically produce only 2 fawns/litter. To overcome this hurdle, the objective of this study was to identify an easily obtainable marker that could be used to correlate an effective dose of 20,25-diazacholesterol with contraceptive efficacy. This would then permit us to quickly estimate the extent of 20,25-diazacholesterol absorption and associated contraceptive efficacy for formulations, dose levels, dosing intervals, etc., by monitoring levels of the biomarker shortly after the initiation of the treatment.

MATERIALS AND METHODS

Chemicals. 20,25-Diazacholesterol was obtained from G.D. Searle Inc. (Peapack, NJ). The purity (>98%) of 20,25-diazacholesterol was confirmed by elemental analysis, melting point analysis, mass spectrometry, and nuclear magnetic resonance spectroscopy (*5*). Desmosterol and cholesterol analytical standards were obtained from Sigma Chemical (Milwaukee, WI). Heptanesulfonic acid solution (IPC B7) was obtained from Alltech Associates (Deerfield, IL). Progesterone and testosterone radioimmunoassay (RIA) kits were obtained from Diagnostic Products Corp. (Los Angeles, CA).

Animals. Coturnix quail eggs were purchased from GQF Manufacturing (Savanah, GA) and hatched in an incubator. Chicks were raised in a brooder and fed Game Bird Maintenance Chow (Purina, Gray Summit, MO). After raising the quail to sexual maturity (>42 days old), 40 male and 40 female quail were randomly selected for the experiment.

Game bird maintenance chow was fortified with 0.1% diazacholesterol (w/w) and fed to 20 male and 20 female quail for 2 weeks. Control male and female birds were fed maintenance chow without 20,25-diazacholesterol. The dosed quail were subsequently paired with untreated quail of the opposite sex. This 20,25-diazacholesterol treatment reduced egg production by 80−90% (*6*). Using a 500 μL syringe containing 10 μL of 7.5% EDTA solution, approximately 0.5 mL of blood was drawn from the jugular vein of each bird every 7 days, beginning 3 weeks prior to treatment and ending 8 weeks posttreatment initiation. Plasma was prepared from blood by centrifugation at 16 100*g* for 3 min. Plasma samples were stored at −20 °C until analyzed for 20,25-diazacholesterol, cholesterol, and related biochemicals.

Deer were obtained from a captive herd at Pennsylvania State University, University Park, PA. Deer were contained in a 17 acre fenced facility containing several paddocks to facilitate separation of treatment groups. Deer were provided ad libitum access to hay and standard feed pellets throughout the study. Three treatment groups consisting of six female deer each were fed 20,25-diazacholesterol in 50 mL of molasses. Each deer received a dose of 0, 1, or 10 mg of 20,25-diazacholesterol/(kg of bodyweight/day) for 10 consecutive days. Deer were artificially inseminated at next estrus. Blood was collected pretreatment, 10 days, 1 month, and 4 months after the initiation of the experiment. Plasma preparation and storage procedures were identical to those used for quail blood.

Table 1. HPLC Parameters

	20,25-diazacholesterol	cholesterol, desmosterol
column	250 × 3 mm C18 ODS/H Keystone (Bellefonte, PA)	250 × 4.6 mm C18 Keystone Valuepak
mobile phase	0.005 M heptanesulfonic acid in H_2O–ACN (70:30)	2-propanol–acetonitrile (77.5:22.5)
flow rate	0.5 mL/min	0.5 mL/min
injection vol	20 μL	20 μL
detector	UV @ 206 nm	UV @ 206 nm
oven temp	ambient	ambient
run time	30 min	25 min

Sample Preparation. The high-performance liquid chromatography (HPLC) method of Johnston et al. (*5*) was used for the analysis of 20,25-diazacholesterol in blood plasma. The high-performance liquid chromatography method of Kim and Chung (*7*) was used for the analysis of free cholesterol, cholesterol esters, and related biochemicals in blood. An aliquot (50 μL) of plasma was transferred into a 1.5 mL plastic eppendorf tube. A 100 μL aliquot of 2-propanol:acetonitrile mobile phase was added to the tube. The tube was capped, vortex mixed for about 5 s, and centrifuged at 14000*g* for 3 min. The supernatant was then transferred to a HPLC autosampler vial containing a 100 μL glass insert and analyzed for 20,25-diazacholesterol or desmosterol using the appropriate HPLC conditions.

HPLC Analyses. The samples were analyzed on a Hewlett-Packard 1090 HPLC using the conditions in **Table 1**. Quality-control samples were prepared by fortifying chicken or bovine plasma (Sigma Chemical) with 20,25-diazacholesterol or desmosterol. Low-level fortified samples contained a 20,25-diazacholesterol concentration of 35 μg/mL or desmosterol and cholesterol concentrations of 60 and 13.4 μg/mL, respectively. High-level fortified samples contained a 20,25-diazacholesterol concentration of 150 μg/mL or desmosterol concentration of 300 and 67 μg/mL, respectively. Low, high, and control QC samples were analyzed at the beginning and end of each batch of plasma samples. A method limit of detection (MLOD) was calculated for each analysis lot. The MLOD was calculated as the concentration of compound required in plasma to generate a signal equal to 3 times the baseline noise at the appropriate retention time in the control plasma chromatogram. This concentration was calculated using the average analyte and baseline noise responses of the low-level and control QC samples for each analysis day.

Gas Chromatography/Mass Spectrometry. In an attempt to identify metabolites of 20,25-diazacholesterol in plasma, 1 μL aliquots the samples in ACN:IPA were analyzed by gas chromatography/mass spectrometry using a Hewlett-Packard 5890 gas chromatograph equipped with a 5970 mass selective detector. The GC was equipped with a 0.250 mm × 30 m DB-5 column (J&W Scientific, Folsom, CA) and operated with helium carrier gas at a linear velocity of 39 cm/s (approximately 1 mL/min). The oven temperature was ramped from 175 to 295 °C at 15 °C. The oven was then maintained at 295 °C for 16.5 min. The injector temperature was 300 °C, and the source temperature was 280 °C. Mass spectrometry conditions were as follows: ionization energy, 70 eV; mass range, *m*/*z* 50−550.

Radioimmunoassay. Progesterone and testosterone levels were determined in blood plasma by following the directions in each radioimmunoassay (RIA) kit. A 100 μL aliquot of plasma was combined with 1 mL of I^{125} progesterone or I^{125} testosterone and incubated at room temperature for 3 h in tubes coated with the appropriate antibodies. The contents of the tubes were decanted, and the remaining I^{125} was quantified using a Genesys 5000 series multiwell γ counter (Laboratory Technologies, Inc., Shaumburg, IL) (*6*).

Statistical Analyses. Pairs of treatments were analyzed using the Student *t*-test. Multiple treatments were compared using analysis of variance (ANOVA) (*8*). Mean desmosterol concentrations and associ-

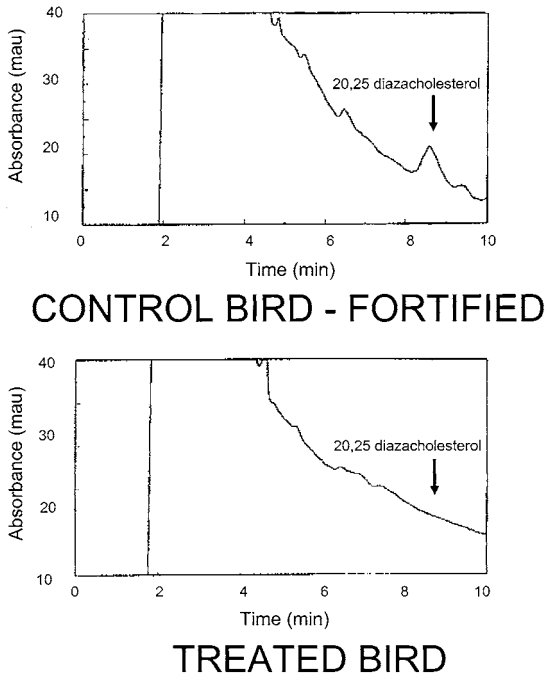

Figure 2. Chromatographic analysis of 20,25-diazacholesterol-fortified control plasma and plasma from quail treated with 20,25-diazacholesterol.

ated standard deviations in quail and deer plasma were calculated using MS Excel (*9*).

RESULTS AND DISCUSSION

20,25-Diazacholesterol Levels in Blood Plasma. As the goal of this study was to identify a biomarker that could be correlated to an effective dose of 20,25-diazacholesterol, it was imperative to analyze blood plasma from birds dosed with an efficacious amount of 20,25-diazacholesterol. On the basis of the approximate feed consumption rates, it was determined that plasma was collected from male and female birds dosed at an average concentration of 100 mg/(kg of bodyweight/day). The quantity of viable eggs produced by the treated groups was 80−90% less than the control groups ($P < 0.001$) (*6*). These results indicated blood plasma collected from these birds would be a valid matrix for identifying biomarkers related to an efficacious dose of 20,25-diazacholesterol.

The obvious initial candidate for a marker compound was the parent, 20,25-diazacholesterol. Johnston et al. developed a chromatographic separation method that used ion-pairing between 20,25-diazacholesterol and hepatanesulfonic acid (*5*). This approach was used to quantify 20,25-diazacholesterol in plasma prepared from control and treated birds. With each set of 10 samples, a blank and plasma samples fortified with 20,25-diazacholesterol at 35 and 150 μg/mL were analyzed. The mean recoveries for the low and high fortified plasma ranged from 75% to 83%, respectively. Daily MLODs averaged 17 μg/mL. However, the analysis of blood plasma collected from the dosed birds failed to detect any 20,25-diazacholesterol (**Figure 2**). This suggested that 20,25-diazacholesterol was not a suitable biomarker for our purposes. Additionally, no other novel chromatographic responses were detected in the samples collected from 20,25-diazacholesterol-treated birds.

Analysis of Sterol Concentrations. As cholesterol is a precursor for many sex hormones, we analyzed blood plasma samples for free cholesterol and similar compounds using reversed phase HPLC and GC/MS methodology (*4*, *7*). Due to the relatively nonpolar nature of these analytes, an atypical HPLC mobile phase of isopropyl alcohol and acetonitrile was used with a microbore C18 column (**Table 1**). HPLC chromatograms of blood plasma from treated birds indicated the presence of two novel compounds in plasma from birds fed 20,25-diazacholesterol (**Figure 3**). Both compounds had a shorter retention time than cholesterol, suggesting that these compounds were more polar than cholesterol. Both compounds were detectable in plasma collected 1 week after 20,25-diazacholesterol administration through week 20 of the study (17 weeks after termination of 20,25-diazacholesterol treatment). These findings suggest that the unknown compounds had excellent potential as biomarkers. Additionally, it was noted that free cholesterol levels were lower in birds treated with 20,25-diazacholesterol.

Identification of Induced Sterols. The plasma extracts containing the highest levels of the two candidate biomarkers were analyzed by GC/MS. The mass spectra of these two compounds were virtually identical, indicating that both potential

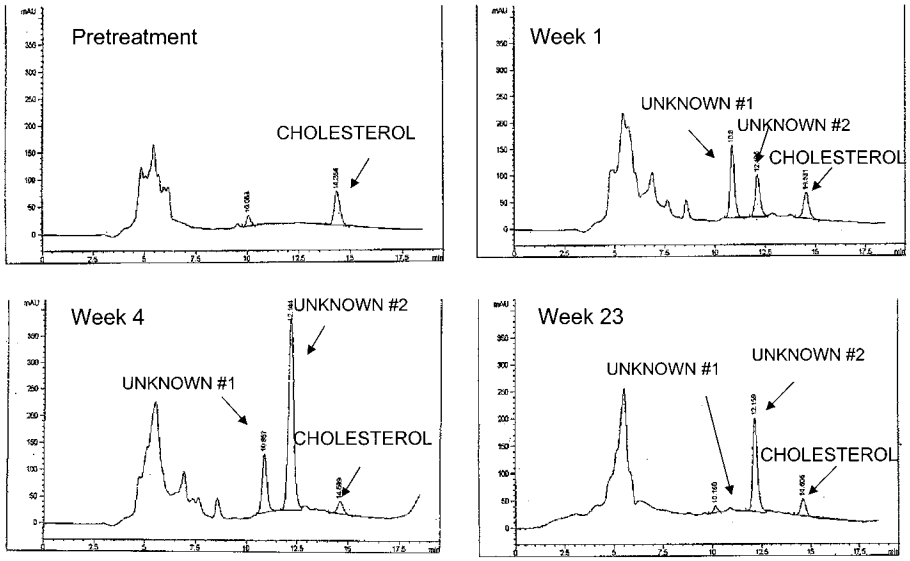

Figure 3. Sterol analysis of blood plasma from quail treated with 20,25-diazacholesterol (retention times: unknown no. 1, 10.8 min; desmosterol, 12.1 min; cholesterol, 14.5 min).

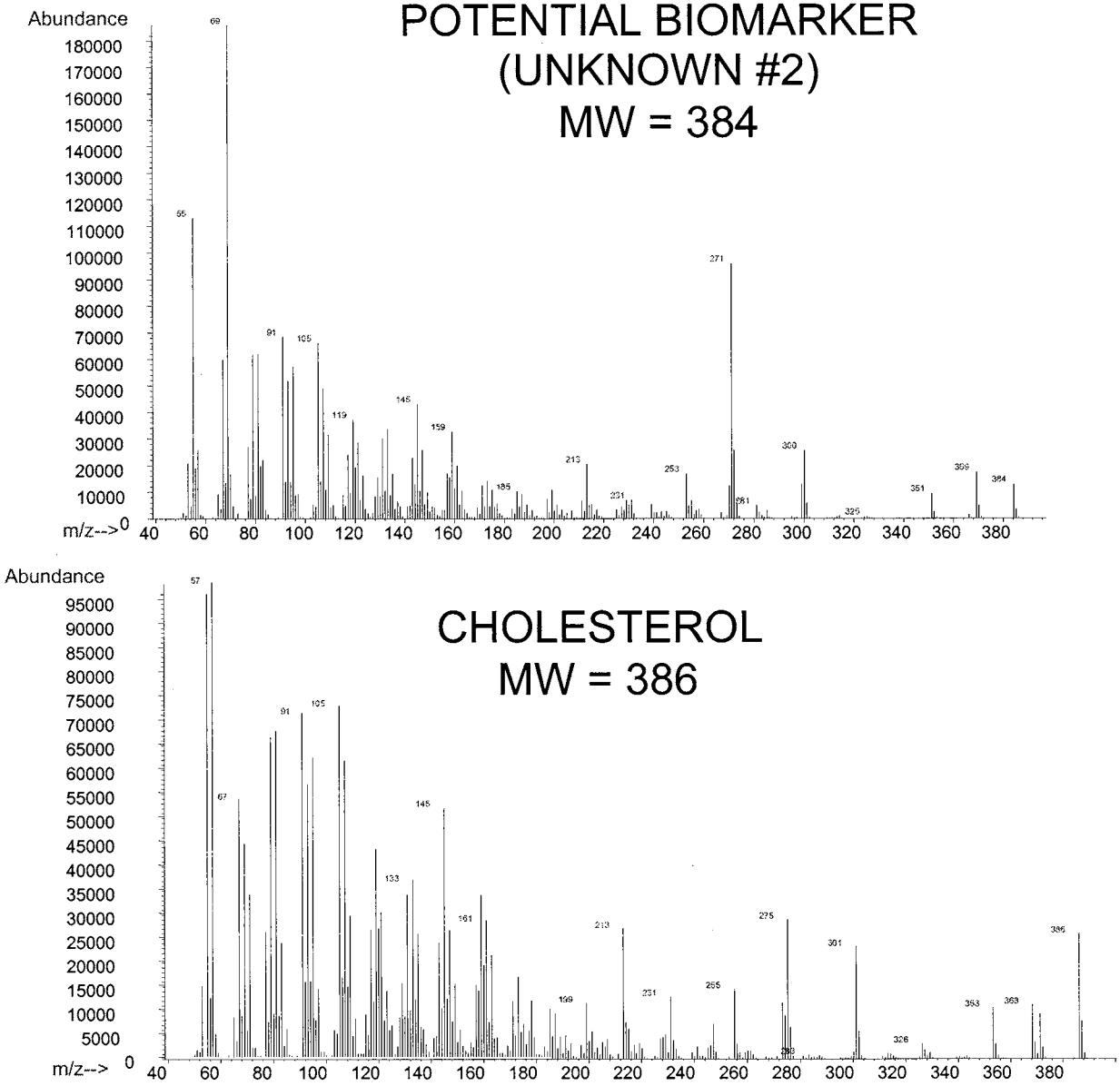

Figure 4. GC/MS analysis of potential biomarker (unknown no. 2, compound with longer retention time) for 20,25-diazacholesterol treatment.

markers had molecular weights of 384 (**Figure 4**). As the mass spectra of these two unknowns were similar to the mass spectra of cholesterol and treated birds had lower plasma levels of cholesterol, we presumed that the potential biomarkers were precursors of cholesterol. A review of the biosynthesis of cholesterol (**Figure 5**) indicated that cholesterol precursors with molecular weights of 384 include desmosterol and 7-dehydrocholesterol (*10*). Standards for these compounds were obtained (Aldrich Chemical) and analyzed by HPLC and GC/MS. The desmosterol standard coeluted with unknown no. 2 (the potential biomarker with the longer retention time). Additionally, the computer generated mass spectra match between the desmosterol and unknown no. 2 spectra was greater than 95%. These analyses indicated that the potential biomarker compound with the longer HPLC retention time was desmosterol. As indicated in **Figure 3**, desmosterol was not detected in the pretreatment blood plasma. For many birds, the desmosterol (unknown no. 2) peak was easily resolved in the HPLC chromatogram of blood collected from birds after 1 week of feeding on 1% 20,25-diazacholesterol feed. By week 4, desmosterol plasma levels from treated birds were greater than control levels (**Figure 6**). Desmosterol levels increased through week 7 and remained elevated for the remainder of the 23 week study, even though the birds were switched to a control diet after 2 weeks of feeding the 20,25-diazacholesterol-fortified feed. Variability in desmosterol levels would likely be less in treatment groups fed a controlled dose of diazacholesterol-fortified feed compared to the ad libitum consumption of fortified feed used in this study. These results indicate that desmosterol would be a suitable biomarker for monitoring consumption of 20,25-diazacholesterol by birds.

Progesterone and testosterone were also evaluated as potential biomarkers. While the levels of these hormones were effected by administration of 20,25-diazacholesterol, the relative standard deviations (RSD) for the desmosterol analyses were 15−30% lower than the RSD-observed hormone levels. These findings suggest that desmosterol would be a better biomarker than progesterone or testosterone.

These results also indicate that 20,25-diazacholesterol inhibits cholesterol synthesis via a Triparanol-like mechanism (*11*, *12*). This mechanism involves the inhibition of the enzymes capable of reducing the double bond at the 24 position of dehydrocho-

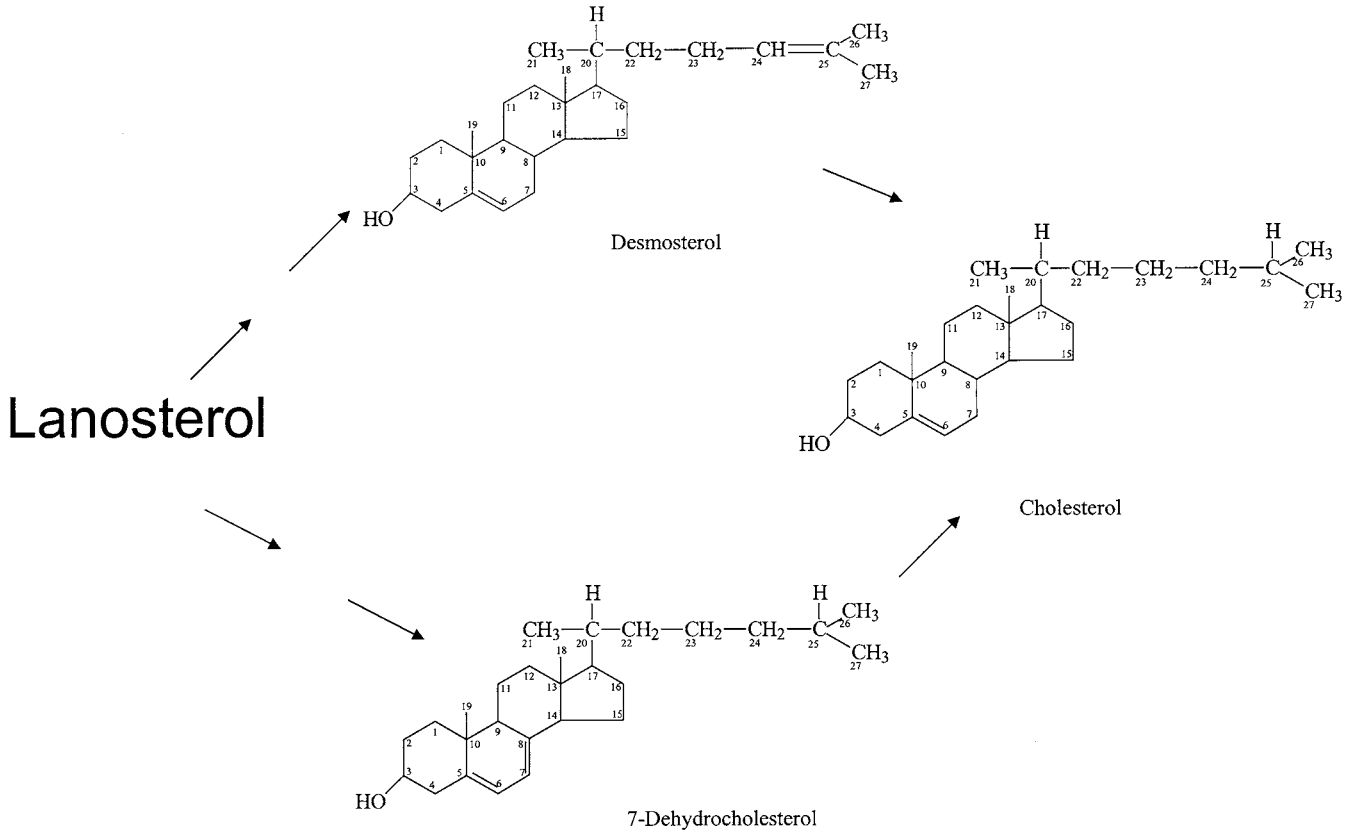

Figure 5. Cholesterol precursors with molecular weights of 384.

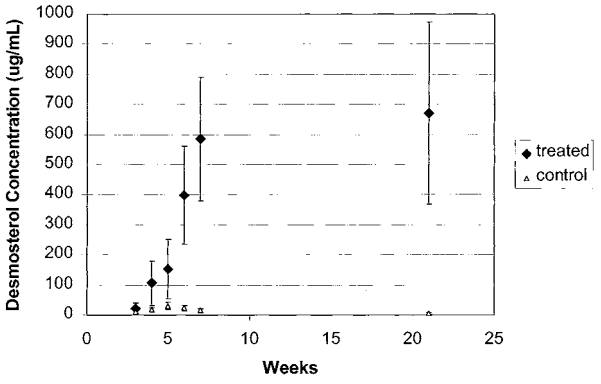

Figure 6. Mean (± standard deviation) desmosterol concentrations in quail plasma.

lesterol in the production of cholesterol. Such an enzyme would likely be involved in other steps in the cholesterol biosynthesis pathway. As illustrated in **Figure 7**, the conversions of cholest-7,24-diene-3β-ol to cholest-7-en-3β-ol and cholest-5,7,24-trien-ol to 7-dehydrocholesterol require the reduction of the double bond at the 24 position. Inhibition of the required enzyme would likely result in elevated levels of cholest-7,24-diene-3β-ol and cholest-5,7,24-trien-ol. Of these two compounds, the chromatographic retention of the diene is expected to be more similar to desmosterol than the triene. Additionally, the molecular weight for cholest-7,24-diene-3β-ol is 384, while the molecular weight of cholest-5,7,24-trien-ol is 382. It is likely that cholest-7,24-diene-3β-ol is responsible for the other chromatographic peak observed in the plasma extract chromatogram of 20,25-diazacholesterol-treated birds.

Cholest-7,24-diene-3β-ol has been identified in the hamster spermatozoa (*13*). During maturation, decreased cholesterol levels were accompanied by increased levels of desmosterol and cholest-7,24-diene-3β-ol. The authors hypothesized that in hamster caudal epididymal spermatozoa, the final step of cholesterol biosynthesis (reduction of the C-24 double bond) is blocked, resulting in an accumulation of desmosterol and cholest-7,24-diene-3β-ol. When propagated in a medium containing compounds which strongly inhibited the C-24 sterol reductase enzyme system, cholest-7,24-diene-3β-ol was detected in the nematode *Caenorhabditis elegans* (*14*). As cholest-7,24-diene-3β-ol is not available commercially and the chromatographic peak areas for the analysis of plasma extracts were greater for desmosterol than for the tentatively identified cholest-7,24-diene-3β-ol, additional effort regarding the positive identification of unknown no. 1 was unwarranted.

The applicability of desmosterol as a biomarker for the development of 20,25-diazacholesterol-based contraceptive for pest deer was evaluated by analyzing plasma from white-tail deer which were fed 20,25-diazacholesterol-fortified molasses at 1 mg/(kg/day) and at 10 mg/(kg/day). The reproduction rates for the 1 mg/kg (1.2 fawns/doe) and control (1.3 fawns/doe) groups were not significantly different ($p = 0.667$). However, the reproduction rate in the 10 mg/(kg/day) treatment group (0.3 fawns/doe) was significantly less than the control ($p = 0.029$) and the 1 mg/(kg/day) ($p = 0.030$). These efficacy results suggest that 20,25-diazacholesterol is capable of reducing the reproductive rate in white tailed deer, even though higher doses of 20,25-diazacholesterol may be required to achieve optimal contraceptive efficacy. Even at these doses, elevated desmosterol concentrations were detected in the 10 mg/kg treatment group at the end of the 10 day treatment period ($p = 0.010$) (**Figure 8**). Elevated desmosterol concentrations were detected in the 1 mg/kg and 10 mg/kg treatment groups at day 30 (20 days

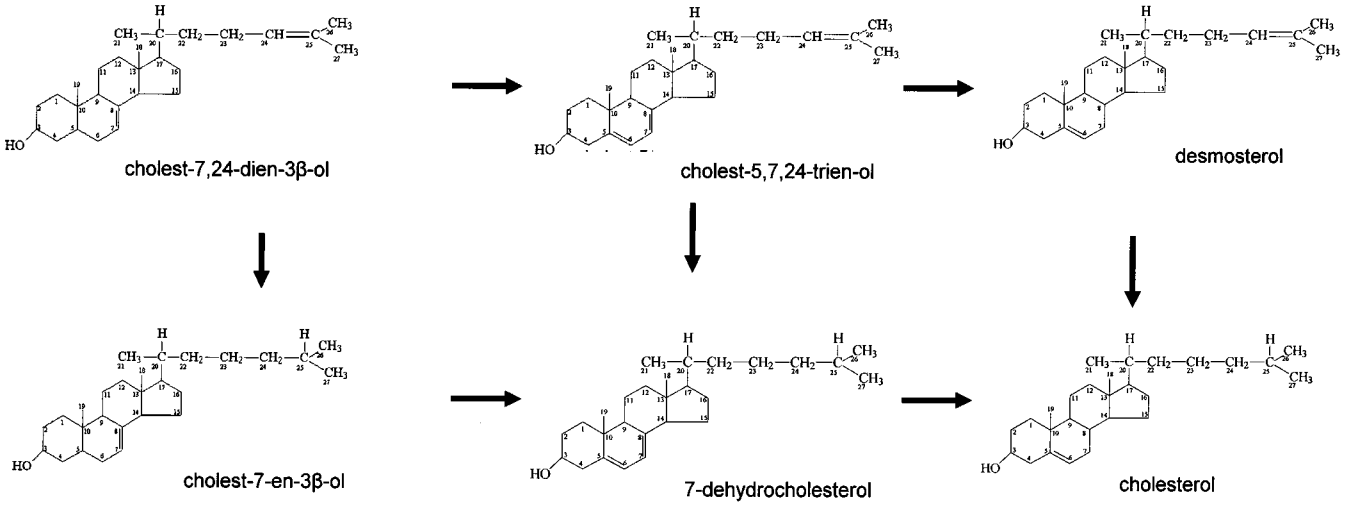

Figure 7. Biosynthesis of cholesterol.

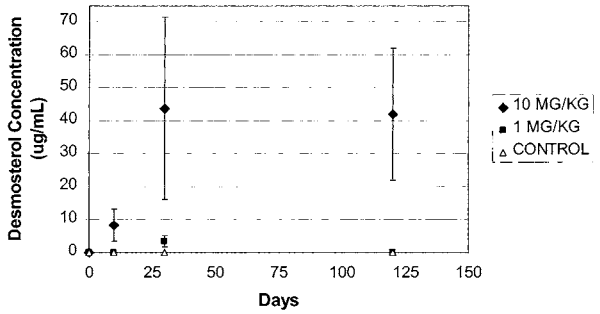

Figure 8. Mean (± standard deviation) desmosterol concentrations in deer plasma.

posttreatment). At this time point, the mean plasma desmosterol level was greater ($p = 0.029$) in the 10 mg/kg dosed animals than in the 1 mg/mL dosed animals. These data suggest a dose−response relationship between desmosterol plasma concentrations and 20,25-diazacholesterol administration. For the 10 mg/kg treatment group, desmosterol levels remained elevated throughout the 4 month study duration ($p = 0.004$).

A comparison of desmosterol and progesterone levels indicated that in response to 20,25-diazacholesterol treatment, desmosterol levels responded more quickly than did progesterone levels. Desmosterol was a better indicator of 20,25-diazacholesterol absorption in the first 1 month postdosing. Thereafter, both compounds appear to be equally feasible as a marker compound. For screening and research purposes, it is preferable to evaluate the effectiveness of a dose as quickly as possible. For this reason, we chose to pursue desmosterol rather than progesterone as a marker for the development of 20,25-diazacholesterol-based contraceptive for pest wildlife.

These studies indicate that desmosterol would be a suitable marker for the determination of 20,25-diazacholesterol absorption in avian and mammalian wildlife. Additional studies are required to determine the optimal doses required to achieve desired levels of contraceptive efficacy. Determination of the accompanying blood levels of desmosterol will then permit researchers to quickly develop and evaluate formulations and dosing procedures to permit the use of 20,25-diazacholesterol as an socially acceptable, effective tool for the management of pest wildlife populations.

LITERATURE CITED

(1) Fagerstone, K. A.; Clay, W. H. An overview of United States Department of Agriculture Animal Damage Control efforts to manage over abundant deer. *Wildl. Soc. Bull*. **1997**, 25, 413−417.

(2) Counsell, R. E.; Klimstra, P. D.; Ranney, R. E.; Cook, D. L. Hypocholesterolemic agents. I. 20-alpha (2-Dialkylaminoethyl)-aminopregn-5-en-3beta-ol derivatives. *J. Med. Chem*. **1962**, *5*, 720−729.

(3) Counsell, R. E.; Klimstra, P. D.; Nystad, L. N.; Ranney, R. E. Hypocholesterolemic agents. V. Isomeric azacholesterols. *J. Med. Chem*. **1965**, *8*, 45−48.

(4) Counsell, R. E.; Lu, M. C.; Masry, S. E.; Weinhold, P. A. Inhibition of cholesterol side chain cleavage by azacholesterols. *Biochem. Pharmacol*. **1971**, *20*, 2912−2915.

(5) Johnston, J. J.; Goodall, M. J.; Hurley, J. C.; Yoder, C. A.; Miller, L. A. Determination of DiazaCon in Quail Feed and Quail Serum by Ion Pair Reversed-Phase Chromatography. *J. AOAC Int.* **2001**, *84*, 634−639.

(6) Yoder, C. A. Use of 20,25-diazacholesterol AgnRH, and cRCP to inhibit reproduction in Coturnix quail. Masters of Science Thesis, Department of Fishery and Wildlife Biology, Colorado State University, Fort Collins, CO, 2000.

(7) Kim, J.; Chung, T. Direct determination of free cholesterol and individual cholesteryl esters in serum by high-pressure chromatography. *Korean J. Biochem.* **1984**, *16*, 69−77.

(8) Anderson, R. L. *Practical Statistics for Analytical Chemists*; Van Nostrand Reinhold Co.: New York, 1987.

(9) Microsoft Corp. *Microsoft Excel*; Microsoft Corp.: Redmond, WA, 2002.

(10) Lehninger, A. L. *Biochemistry, The Molecular Basis of Cell Structure and Function*; Worth Publishers: New York, 1975.

(11) Thompson, M. J.; Dupont, J.; Robbins, W. E. The sterols of liver and carcass of 20,25-diaza-colesterol fed rats. *Steroids* **1963**, *2*, 99−104.

(12) Ranney, R. W.; Cook, D. L.; Hamborer, W. E.; Counsell, R. E. An azasterol that induces hypocholesterolemia in rats. *J. Pharmacol. Exp. Ther*. **1963**, *142*, 132−136.

(13) Awano, M.; Kawaguchi, A.; Morisaki, M., Mohri, H. Identification of Cholesta-7,24-dien-3β-ol and desmosterol in hamster cauda epididymal spermatozoa. *Lipids* **1989**, *24*, 662−664.

(14) Chitwoood, D. J.; Lusby, W. R.; Lozano, R.; Thompson, M. J.; Svoboda, J. A. Sterol Metabolism in the Nematode Caenorhabditis elagans. *Lipids* **1984**, *19*, 500−506.

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