Determination of 17β-Hydroxy-7α,17-dimethylestr-4-en-3-one (Mibolerone) in Canned Dog Food by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic method (LC) is described for the assay of mibolerone in four flavors of canned dog food products at the 163 ppb level. Mibolerone is extracted from the dog food with UV grade acetonitrile, partitioned between UV grade hexane and deionized water, then partitioned between UV grade hexane and methanol–water, and finally between methanol–water and methylene chloride. The residue upon drying is treated with 2:1 Celite–Nuchar SN active carbon and chromatographed on a silica gel column. The eluate after removal of the solvent is dissolved in 55:45 UV grade acetonitrile–water and chromatographed on a 4.6 mm (i.d.) × 25 cm RP-8-10A LiChrosorb column with the same solvent as the mobile phase. Observed recovery was 95.7 ± 4.5% for mibolerone in canned dog food.

Recent interest in the control of the pet population has led to the investigation of estrus inhibitor drugs in the canine bitch (Sokolowski, 1974; Sokolowski and Zimbelman, 1976). Mibolerone (17β-hydroxy-7α,17-dimethylestr-4-en-3-one) is an anabolic, androgenic, nonprogestational, nonestrogenic steroid. Given orally on a daily basis it effectively inhibits estrus in the bitch.

One route of administration is via canned dog food products. Consequently, a precise method was needed to monitor the level of the drug in various dog food formulations (flavors) with a required sensitivity of 163 ppb (30 μg/6.5-oz can).

A double antibody radioimmunoassay (RIA) developed in our laboratory was available. However, it lacked the required precision and was considered too lengthy for the intended use. An alternate method was devised based on rigorous cleanup, followed by quantitation with a high-performance liquid chromatograph (LC) that gave the required sensitivity and was 50% faster than the RIA method. This method is described herein.

EXPERIMENTAL SECTION

Apparatus. Waring Blendor, Model 702B, refitted with gaskets cut from polyethylene; a DuPont 820 Liquid Chromatograph, equipped with a precision photometer (sensitivity of 0.005 absorbance units full-scale deflection at 254 nm); 4.6 mm i.d. × 25 cm RP-8-10A column, Anspec, Inc.; rotary evaporation unit or equivalent equipment (Gosline et al., 1969); Loop Injector, CV-6-HPAX, 3000 psi valve and 50-CV6 loop, Valco, Instrument Co., Adapter, 24/40, No. 5225-10, Ace Glass, Inc.

Reagents and Solutions. Mibolerone standard, 99.2% purity (17β-hydroxy-7α,17-dimethylestr-4-en-3-one), The Upjohn Company; primary stock solution was prepared in acetone; dilute standards were prepared from aliquots of stock standard that were nitrogen evaporated followed by dissolution in UV acetonitrile; silica gel 60 (70-230 mesh) ASTM, E. M. Laboratories; anhydrous sodium sulfate, washed with chloroform and oven-dried at 110 °C; sodium sulfate solution, prepared from chloroform-washed sodium sulfate; Celite-NuChar SN, mixed dry (2:1 ratio), John Mansville and Westvaco Chemical Division, respectively.

Solvents. Burdick and Jackson Laboratories, distilled-in-glass grade for all solvents; hexane and acetonitrile were UV grade.

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Extraction of Canned Dog Food. The entire contents (Figure 1) of a 184-g (6.5 oz) can of dog food were placed into a glass Waring Blendor bowl. Two-hundred milliliters of UV acetonitrile and 50 g of anhydrous sodium sulfate were added, and the mixture was blended for 3 min at low speed. A 350-mL sintered glass funnel (medium porosity) with a 50-g layer of Celite was washed with acetonitrile. The mixture was filtered with vacuum into a clean suction flask. The extraction and filtration steps were repeated two more times with 150 mL of UV acetonitrile. The combined acetonitrile extracts were transferred to a 1-L mixing cylinder and diluted to 1.0 L. A one-quarter aliquot (250 mL) was transferred to a 1-L round-bottom flask. After addition of 50 mL of benzene, a glass trap (Adapter No. 5225-10) was attached and the solvent rotary evaporated with the aid of a water bath at 40 to 45 °C (Gosline et al., 1969). *Caution: Bumping may occur.* Addition of 50-mL volumes of benzene periodically during the evaporation step minimizes the bumping and aids in the removal of the water.

Hexane–Water Partition. The oily residue was transferred quantitatively to a 500-mL separatory funnel with two successive 100-mL portions of UV hexane. The first 100 mL of UV hexane was used to rinse the glass adaptor. Twenty-five milliliters of deionized water was added and the separatory funnel was shaken vigorously for 1 min. The phases were allowed to separate. The lower layer was drained back into the round-bottom flask and the hexane layer was decanted into a 1-L separatory funnel. The partition step was repeated a second and third time with 100 mL and 50 mL of hexane, respectively. The hexane layer was decanted into the separatory funnel each time.

Hexane Methanol–Water Partition, Methylene Chloride Extraction. The three combined hexane phases (350 mL) were partitioned with four 100-mL volumes of 9:1 methanol–deionized water. The funnel was shaken vigorously for 1 min, and the phases were allowed to separate completely. The lower layer from each partition step was drained into a clean 1-L separatory funnel.

One hundred and fifty milliliters of deionized water, 1.0 mL of saturated sodium sulfate solution, and 250 mL of methylene chloride were added to the combined methanol–water phases (400 mL) in the second 1-L funnel. The separatory funnel was shaken vigorously for 1 min. *Caution: Extreme pressure buildup made venting necessary throughout the shaking period.* The phases were allowed to separate completely and the lower layer drained into a 1-L round-bottom flask. The extraction was re-

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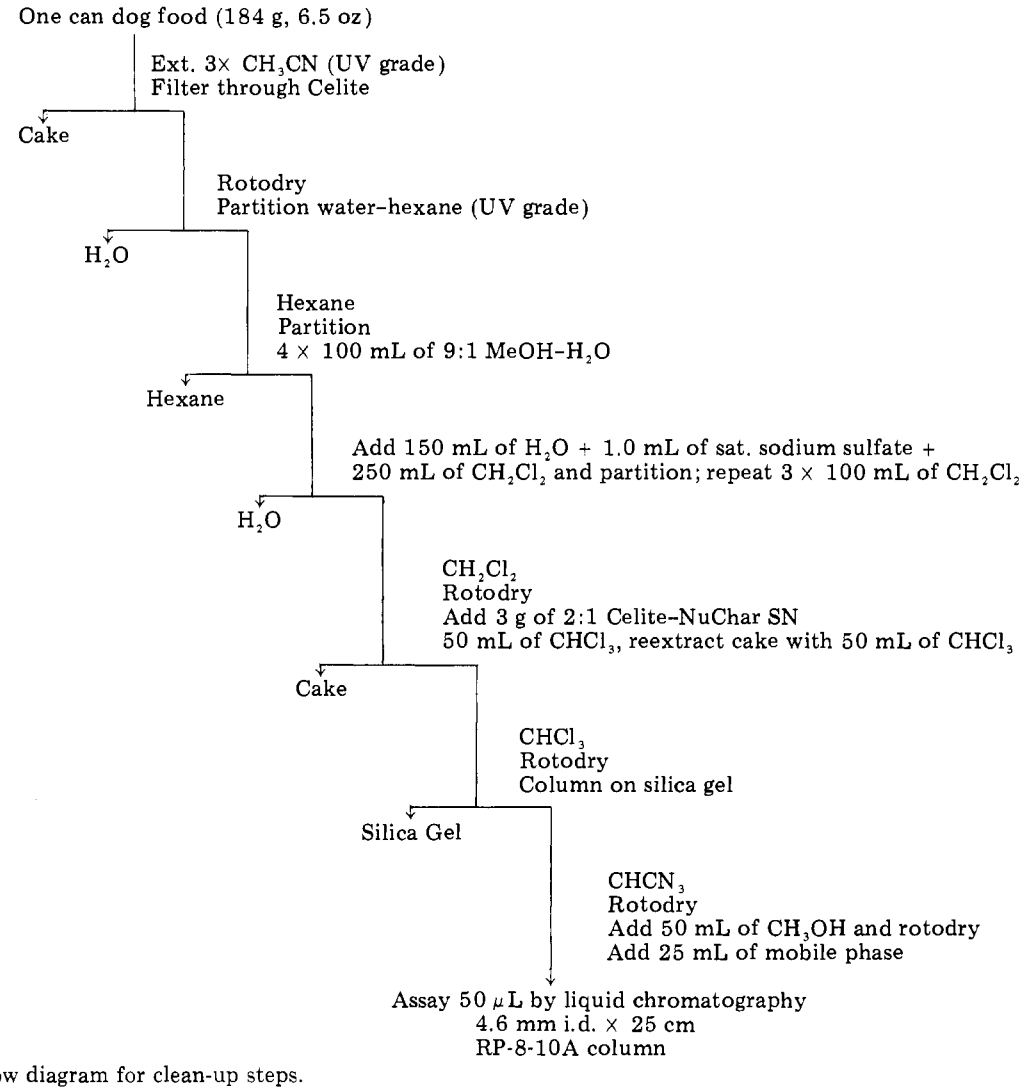

Figure 1. Flow diagram for clean-up steps.

peated three more times with 100-mL portions of methylene chloride. Fifty to 100 mL of benzene was added to the round-bottom flask and rotary evaporated. When dry, 50 to 100 mL of methanol was added, and the solvent was again rotary evaporated.

Celite–NuChar SN Cleanup. Three grams of 2:1 Celite–NuChar SN and 50 mL of chloroform were added to the dried residue in the round-bottom flask from the preceding operation. It was shaken on a wrist action shaker for 5 min at a medium rate. The carbon mixture was filtered through a 150-mL medium porosity fritted funnel with vacuum. The round-bottom flask and charcoal filter cake were washed with 2 × 25-mL portions of chloroform. The charcoal cake was transferred to the round-bottom flask, and the shaking, washing, and filtration step was repeated. The filtrate (chloroform solution) was transferred to a clean 500-mL round-bottom flask and rotary evaporated to dryness.

Silica Gel Chromatography. A slurry of 25 g of Silica Gel 60 and 100 mL of 1:9 ethyl acetate–benzene was poured into a 20 mm × 40 cm glass chromatographic column and tapped to settle the particles. A plug of glass wool was placed on top of the silica gel and a 250-mL reservoir fitted to top of the column.

The column was washed with 100 mL of 1:9 ethyl acetate–benzene. The sample was transferred to the column with three 4.0-mL portions, followed by one 10.0-mL portion of the 1:9 mixture. Three-hundred milliliters of the 1:9 mixture was swirled in the sample flask and added to the top of the column. Mibolerone was eluted with 150 mL of UV acetonitrile into a 500-mL round-bottom flask. Nitrogen pressure was used to completely blow the liquid out of the column and into the flask. The eluate was evaporated to dryness. Fifty milliliters of methanol was added to the round-bottom flask, swirled, and again rotary evaporated.

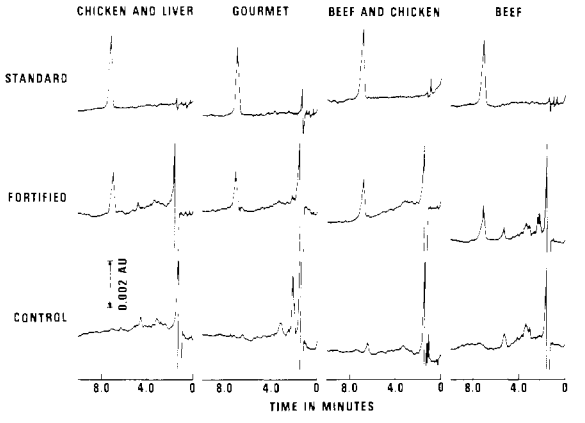

Figure 2.

Liquid–Liquid Chromatography Conditions. The DuPont 820 was adjusted so that: first stage air was 50 psi, second stage air was 12-14 psi, i.e., the flow rate was approximately 1.0 mL/min at 500 psi column pressure and the UV detector was set at 0.02 absorbance units full scale.

Table I. Percent Recovery of Mibolerone from Fortified Canned Dog Food[a]

Mibolerone, μg		Percent		
Added	Found	Recovery	Av ± SD	CV
		Chicken and Liver Flavor		
0	0	0		
30	29.1	97.0		
30	27.9	93.0		
30	27.9	93.0		
30	30.4	101.2		
30	31.6	105.4	97.8 ± 5.5[b]	5.6
		Gourmet Flavor		
0	0	0		
30	29.7	99.1		
30	28.4	94.6		
30	28.9	96.5		
30	27.6	92.1		
30	27.6	92.1	94.9 ± 3.0[b]	3.2
		Beef and Chicken Flavor		
0	0	0		
30	28.9	96.5		
30	28.9	96.5		
30	28.9	96.5		
30	26.3	87.7		
30	27.6	92.1	93.9 ± 3.9[b]	4.2
		Beef Flavor		
0	0	0		
30	26.3	87.7		
30	30.3	100.8		
30	30.3	100.8		
30	28.3	94.3		
30	28.9	96.5	96.0 ± 5.4[b]	5.6

[a] The contents of each can was fortified directly in the Waring Blendor jar with acetonitrile solution containing 30 μg of mibolerone (0.3 mL of 100 ppm). [b] Overall observed recovery for all *four flavors* was 95.7 ± 4.5% with a CV value of 4.7.

The mobile phase, 55:45 UV acetonitrile–water, was placed in the reservoir and degassed as follows: The vacuum and recycle valve was opened for 3.0 min, then only the vacuum valve was opened for 2.0 min, and finally the vacuum and recycle valve was opened for 1.0 min. The mibolerone eluate from the silica gel column was dissolved in 25.0 mL of mobile phase, and a 50-μL aliquot was injected into the LC column with a loop injector. The peak height of the standard and sample was measured to the nearest 0.5 mm using the baseline technique. The amount of mibolerone was calculated from the peak height of a 0.5 ppm standard in mobile phase injected under identical conditions.

Table II. Percent Recovery of Mibolerone from Medicated Canned Chicken and Liver Flavor Dog Food[a]

Mibolerone, μg		Percent		
Added	Found	of theory	Av ± SD	CV
30	26.3	87.7		
30	26.3	87.7		
30	26.3	87.7		
30	26.3	87.7		
30	28.9	96.5		
30	27.6	92.1	89.9 ± 3.7	4.1

[a] Medication added in 3 mL of propylene glycol solution by the manufacturer and then retorted.

RESULTS AND DISCUSSION

Recovery Studies. Recoveries were based on the amount of mibolerone added to the can of dog food at the level of 30 μg/184-g (6.5 oz) can or (163 ppb). Calculations based on the mibolerone found for the 30-μg level showed recoveries to be: chicken and liver flavor, 97.8 ± 5.5%; gourmet flavor, 94.9 ± 3.0%; beef–chicken flavor, 93.9 ± 3.9%; and beef flavor, 96.0 ± 5.4%. The overall observed recovery ± estimated standard deviation for mibolerone in all flavors of canned dog food was 95.7 ± 4.5% (Table I). Chromatograms for all four flavors were quite clean and free of interfering peaks at the retention time of mibolerone (Figure 2).

Method. Cleanliness of glassware is of extreme importance. All glassware should be thoroughly washed and rinsed with methanol, if cleanliness is in doubt. A silica gel column profile should be run to insure proper elution of the mibolerone with the solvent ratios specified in the chromatography section.

Medicated Dog Food Product. Chicken and liver flavor medicated dog food product was also assayed (Table II). These were fortified with 3 mL of propylene glycol solution (10 μg/mL) per can of dog food and retorted under normal commercial methods. The mibolerone values found were slightly lower [2 μg/can less than the theoretical (30 μg/can)]. Information furnished by the manufacturer attributed the loss to the retortion.

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