Determination of Medroxyprogesterone Acetate (6α-Methyl 17α-Acetoxyprogesterone) in Animal Feed Supplements

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An ultraviolet spectrophotometric and a shorter gas chromatographic method are described for the quantitative analysis of medroxyprogesterone acetate (6α-methyl 17α-acetoxyprogesterone) in animal feed supplements at the level of 90 to 120 mg. per pound. Both procedures involve Soxhlet extraction of the ground sample using chloroform, cleanup of the dried extract on a Nuchar C-190 column, and solvent partition between hexane and 70% aqueous methanol. In the shorter procedure, the solvent is evaporated, the residue redissolved in a suitable solvent, and analyzed by gas-liquid chromatography. When 12 pure medroxyprogesterone acetate samples were analyzed by this procedure, the average recovery was 98.6% with a standard deviation of 5.31%, and a coefficient of variation of 5.38%. In the alternate spectrophotometric procedure, medroxyprogesterone acetate is separated from other interfering compounds as a discrete band on a thin-layer chromatographic plate, eluted, and analyzed. Good correlation was obtained on five medicated supplements analyzed by the two procedures.

Several orally active progestational agents have been found effective in synchronizing the estrous cycle of cattle (Hansel and Melven, 1960). One such compound is 6α-methyl 17α-acetoxyprogesterone or medroxyprogesterone acetate (MAP). Hansel (1961) compared MAP with 6-chloro-Δ^6-dehydro-17-acetoxyprogesterone with respect to estrous synchronization and effect on fertility. Zimbelman (1963) has reported on the minimal effective dose of MAP for control of the estrual cycle of cattle.

Medroxyprogesterone acetate is commercially available for estrous synchronization in cattle under the trade name Repromix (TUCO Products Co., Kalamazoo, Mich.), which is a formulation of MAP with soybean mill feed containing 15 grams of MAP per pound. Repromix is mixed by feed manufacturers with commonly used feed ingredients to make cattle feed supplements containing 90 to 120 mg. of MAP per pound (frequently pelleted).

The composition of these feed supplements varies widely, dependent on the geographical location of the feed manufacturer, mixing capability, availability, market price of ingredients, and several other factors. Blank samples are rarely available to the analyst for determination of background. Therefore, rigorous clean-up steps are necessary to remove all interfering substances before determination of MAP.

This communication describes two analytical procedures which have been developed for the analysis of MAP in feed supplements containing 90 to 120 mg. per pound of MAP. Both involve Soxhlet extraction of a representative ground sample using chloroform, cleanup on an activated charcoal column, and solvent partition to remove nonpolar impurities. In the first procedure, MAP from the residue after solvent partition is separated from other impurities as a discrete band on a silica gel thin-layer chromatographic plate, eluted with a suitable solvent, and determined by ultraviolet spectrophotometry. In the shorter second procedure, the extract after solvent partition is evaporated, the residue dissolved in a suitable solvent, and the concentration of MAP determined by gas-liquid chromatographic analysis.

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EQUIPMENT

Cary Model 15 recording spectrophotometer (Applied Physics Corp., Monrovia, Calif.) and 1-cm. matched silica cuvettes. Micro Tek MT220 gas chromatograph with glass inlet, a hydrogen flame ionization detector, and 1-mv. strip chart recorder. Short wavelength ultraviolet lamp.

PROCEDURE

Extraction. Grind about 400 grams of the sample in a Wiley mill using a 1-mm. screen and mix thoroughly. Accurately weigh a sample calculated to contain 2.5 mg. of MAP and transfer to a Soxhlet extraction thimble. Build up the bulk of the sample by adding washed and ignited sea sand (Fisher Scientific Co.) equal to about one-third the volume of the sample. Mix using a stainless steel spatula. Plug the thimble with glass wool and place in a Soxhlet extractor containing 180 ml. of chloroform A. R. in the receiver flask. (Chloroform A. R. is used throughout the procedure.) Rinse the spatula into the thimble with a few milliliters of chloroform. Connect the condenser and extract for 4 hours. Change the chloroform and continue extraction for another 20 hours with 180 ml. of fresh chloroform. Combine the chloroform extracts and evaporate in a water bath at 60° using a rotary evaporator at reduced pressure.

Preliminary Sample Cleanup on Nuchar Column. For Regular Samples. Slurry 3.5 grams of Nuchar C-190-plus 30-mesh (West Virginia Pulp and Paper Co., New York, N.Y.), in a beaker with *n*-hexane (Burdick & Jackson, distilled). Transfer the mixture into a 14- × 7/16-inch chromatographic column fitted with a coarse sintered glass disk and stopcock containing a plug of glass wool at the bottom. (A wire placed in the column before adding the Nuchar may be moved up and down gently to dispel any entrapped air.) Let the Nuchar C subside and place a small plug of glass wool on top of the Nuchar. Allow the *n*-hexane to drain and pass 50 ml. of chloroform through the column, taking care that neither the hexane nor the chloroform layer do not fall below the top of the charcoal layer. Discard these washings. Place a 250-ml. round-bottomed flask below the column as a receiver. Transfer the dried extract to the column with the aid of 5- to 10-ml. portions of chloroform using a total of about 40 ml. After most of the chloroform has passed through the column,

add an additional 75 ml. of chloroform and collect the effluent in the 250-ml. flask. (The flow rate is not critical.) Evaporate the effluent to dryness in a water bath at 60° using a rotary evaporator at reduced pressure.

For Feeds Containing Higher than 25% Alfalfa or Grasses. Prepare the column as described above using 7.0 grams of Nuchar C-190-plus and a 25- × $^{7}/_{16}$-inch chromatographic tube. Pass 75 ml. of chloroform through the column and discard. Place a 250-ml. round-bottomed flask as the receiver. Transfer the residue to the column as before, using a total of about 40 ml. of chloroform. Let drain and collect the effluent in the flask. After most of the chloroform has passed through the column, pass an additional 125 ml. of chloroform through the column. Evaporate the effluent to dryness in the water bath using a rotary evaporator at reduced pressure.

Solvent Partition. Dissolve the dry extract in 15 ml. of *n*-hexane, previously saturated with methanol, and transfer to a 125-ml. separatory funnel. Rinse the flask with 15 ml. of 70% aqueous methanol and transfer to the separatory funnel. Repeat the operation. Shake the funnel about 20 times, allow the phases to separate, and transfer the lower methanol layer into a second 125-ml. separatory funnel containing 30 ml. of *n*-hexane saturated with methanol. Shake the second separatory funnel about 20 times, allow the phases to separate, and drain the methanol layer into a 250-ml. round-bottomed flask.

Repeat the extraction of the *n*-hexane solution in the first separatory funnel with five additional 30-ml. portions of 70% methanol, washing each portion with the same 30 ml. of *n*-hexane contained in the second separatory funnel.

Evaporate the combined methanol extracts to dryness in the water bath at 60° using a rotary evaporator at reduced pressure.

Dissolve and transfer the residue quantitatively into a 10-ml. volumetric flask using chloroform. Adjust to volume and mix.

Spectrophotometric Determination of MAP. Thin-Layer Chromatography. Prepare 0.3-mm. thick thin-layer plates on 8- × 8-inch glass plates using silica gel GF_{254} (Brinkmann Instruments, Inc.). Activate the dry plates for 1 hour at 100° before using. Scribe parallel lines and divide the working area of the activated plates into five equal parts.

Prepare accurately a standard MAP solution to contain about 1 mg. per ml. in chloroform. (Store in a refrigerator and use within 30 days.)

Accurately pipet two 400-μl. portions (about 100 μg. of MAP) of the sample and spot into two of the five areas on the plate about $1^{1}/_{4}$ inches from the bottom. Similarly spot exactly 100 μl. of the standard into two other areas. Leave one area blank.

Develop the plate in a 60 to 40 mixture of ethylacetate and cyclohexane until the solvent front has traveled to within a half inch of the top of the plate.

Remove the plates from the tank and dry at room temperature for about one-half hour in a well ventilated hood. (Be sure that the plate is thoroughly dry. Use a cold air blower if necessary.) Locate the MAP band with the aid of a short wavelength ultraviolet lamp and scribe parallel lines on either side of the band. Scrape the silica gel from each portion onto a glazed powder paper and transfer to separate 15-ml. medium pore, sintered glass funnels connected to a suitable manifold for filtration under vacuum and with a 10-ml. volumetric flask as the receiver. Elute the MAP using four 2-ml. portions of 95% ethanol mixing the silica gel with a glass rod after each addition of fresh solvent. Adjust the volume to mark with 95% alcohol and mix.

Quantitation by Ultraviolet Spectrophotometry. Scan the ultraviolet spectra of the sample and the standard against the blank using a recording spectrophotometer. Record the absorbances at 242 mμ.

Calculations:

$$\frac{A_u \times C_s \times DF \times 453.6}{A_s \times W} = \text{mg. of MAP/lb.}$$

where

A_u = average absorbance of the sample
A_s = average absorbance of the standard
C_s = milligrams per milliliter of MAP in final standard solution from the plate
DF = dilution factor, which for the above procedure is

$$\frac{10 \text{ ml.} \times 10 \text{ ml.}}{0.4 \text{ ml.}} = 250$$

W = weight of the sample in grams

Gas-Liquid Chromatographic Determination of MAP. The gas chromatographic conditions are as follows.

Column:	1% OV-1 on gas Chrom Q, 100- to 120-mesh packed in a 2-foot long, 6-mm. O.D. glass column and previously conditioned at 275°C.
Temperatures:	Column—225°C. Detector—250°C. Injection port—250°C.
Gas Flows:	Helium—120 ml. per minute Hydrogen—60 ml. per minute Air—280 ml. per minute
Attenuation:	8.0×10^{-10} AMP full scale into a 1-mv. recorder

Under these conditions, MAP has a retention time of about $2^{1}/_{2}$ minutes.

Prepare two standard solutions in chloroform containing 0.25 and 0.125 mg. per ml. of MAP. Inject 5 μl. each of high standard, sample, and low standard in that order. Measure the peak height of each.

Calculations:

$$\left[\frac{0.125\ (U - HS)}{FS - HS} + 0.125\right] \times \frac{10 \times 453.6}{W} = \text{mg. MAP/lb.}$$

where

U = peak height of sample
HS = peak height of half standard (0.125 mg./lb.)
FS = peak height of full standard (0.25 mg./ml.)
W = weight of the sample in grams

Figure 1 is a typical chromatogram of the two standards and a sample.

RESULTS AND DISCUSSION

The ultraviolet spectrophotometric procedure was the first one developed for the analysis of MAP. This method was initially tested on five feed supplements which were fortified in the laboratory with known amounts of MAP in the range of 25 to 200 mg. per pound. The composition of the supplements were as follows.

	%
Feed I	
Dried hay (milled through 0.063 screen) consists of *ca.* 90% brome grass, 2 to 5% alfalfa, remainder unidentified	100
Feed II	
Wheat, ground	68.8
Barley, ground	23.0
Cottonseed meal	8.2
Feed III	
Shelled corn, coarse grind	88.4
Molasses, cane	5.0
Soybean oil meal, 44%	5.0
Limestone, ground	0.5
Dicalcium phosphate (18.5%)	0.5
Trace mineral mix (CCC—10% Zn)	0.05
Vitamin D (Whitmoyer's D_3—3000 ICU per gm.)	0.011
Vitamin A (Whitmoyer's—30,000 I.U. per gm.)	0.015
Salt	0.5
Feed IV	
Wheat middlings	4.8
Corn gluten feed	5.7
Crushed barley	7.5
Brewers' dried grains	12.0
Corn distillers' dried grains	4.5
Dried citrus pulp	3.0
Yellow corn meal	52.6
Cane molasses	7.2
Urea	1.2
Dicalcium phosphate	0.45
Ground limestone	0.45
Salt	0.60
Feed V	
Alfalfa pellets (ground)	100

Results of the analyses are given in Table I.

The corresponding unfortified feeds were also analyzed and showed no significant interference in the ultraviolet spectral region of interest. The standard deviation for this set of samples is 2.85% and the 95% confidence interval is ± 5.83%.

The two methods were compared by analyzing five samples using both methods. Agreement was excellent. The results are given in Table II.

Over 200 samples were analyzed by the gas chromatographic procedure. These represent samples from different feed manufacturers and vary considerably in composition. Few of these samples gave more than one peak on the gas chromatograph, indicating effectiveness of the clean-up steps. No sample had an interfering peak. Since the absolute potency of the samples was unknown, it is not possible to make any claim regarding the accuracy of the procedure on these field samples.

Twelve 2.50-mg. samples of pure MAP were subjected to the gas chromatographic procedure. The results averaged 98.50% of theory with a range of 92.9 to 111.5%. The standard deviation for the assay was 5.31%. Thus, the coefficient of variation was 5.38% and the 95% confidence interval ±11.69%.

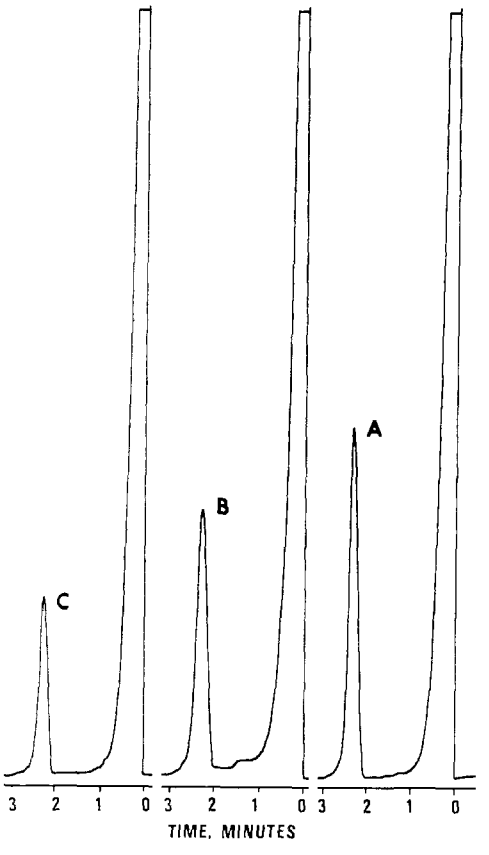

Figure 1. Typical gas chromatographic response curves for medroxyprogesterone acetate obtained under conditions described in the text

A, high standard; B, sample; C, low standard

Table I. Analysis of Blank Supplements Fortified with MAP

Feed Number	Number of Samples Analyzed	Recovery, % of Theory	
		Mean	Range
I	8	101.6	97.9–105.6
II	6	100.9	96.9–104.9
III	3	96.0	95.4– 97.1
IV	4	99.0	92.6–102.8
V	9	98.9	93.7–103.2

Table II. Comparison of Spectrophotometric and Gas-Liquid Chromatography Methods

Number	MAP Found, %	
	Spectrophotometric	GLC
R-193	0.0221	0.0218
R-194	0.0180	0.0184
R-195	0.0183	0.0178
R-196	0.0180	0.0182
R-197	0.0143	0.0146

LITERATURE CITED

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