Determination of Melengestrol Acetate (MGA) Residues in Bovine Tissue

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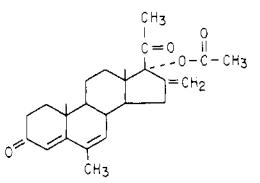
A five-step spectrophotometric method for the determination of the progestogen, melengestrol acetate (MGA), in bovine tissue is described. Samples of muscle, liver, kidney, and tripe are extracted with 95% ethanol and partitioned into chloroform, whereas fat and bone marrow are extracted with hexane and partitioned into 90% methanol. The dried extracts are defatted on silica gel columns, partitioned between 70% methanol and hexane, and further cleaned up by thin-layer chromatography on alumina. The melengestrol acetate zone is scraped from the plate, eluted, and reacted with isonicotinic acid hydrazide solution. The resulting color is measured at 415 mμ. Tissue samples of fat, liver, and muscle fortified at the lowest level of sensitivity (25 p.p.b.) showed that the average recovery of triplicates was 94, 81, and 73%, respectively. Results of residue studies following long-term feeding of beef heifers are reported. This method also can be used to detect progesterone in bovine tissues.

Melengestrol acetate [17-acetoxy-6-methyl-16-methylenepregna-4,6-diene-3,20-dione, or MGA (I)] has been shown by Zimbelman and Smith (1966a, 1966b) to be an effective oral progestational compound whose optimal daily dose for ovulation inhibition in dairy and beef heifers is about 0.4 mg. per animal. Consequently, a tissue residue method was needed that had the combined requirements of low background, good specificity, negligible interference from other steroids, and a sensitivity of 25 p.p.b.

Existing methods for progesterone and related steroids previously reported in the literature either have been used in part or modified until maximum recovery was obtained as indicated by the use of tritium-labeled melengestrol acetate. Such methods include those of Loy *et al.* (1957) for the purification and quantitative estimation of progesterone from luteal tissue; of Goldzieher *et al.* (1961) for the quantitative extraction and partial purification of steroids in tissue and blood; of Quesenberry and Ungar (1964) for thin-layer chromatographic systems for adrenal corticosteroids; and of Umberger (1955) on isonicotinic acid hydrazide as a colorimetric reagent for the determination of Δ^4-3-ketosteroids.

This paper describes the necessary cleanup steps to detect melengestrol acetate in fat, muscle, liver, kidney, tripe, and bone marrow at levels as low as 25 p.p.b.



(I) Melengestrol acetate (MGA)

MATERIALS

Animal Studies. Groups of heifers were fed, for 101 to 279 days, a daily diet fortified at various levels with melengestrol acetate. At the end of this period, they were placed on a nonfortified diet for various periods (0.5 to 7 days) prior to slaughter. Tissue samples were taken at time of slaughter and immediately frozen. Control animals were fed a nonfortified diet and handled in the same manner as the treated groups.

Apparatus. A Lourdes tissue homogenizer with 300-ml. stainless steel blender cups with silicone rubber gaskets is recommended. Lubrication of bushings was avoided. To reduce wear of cup-heat and shaft bushings, glass fiber washers cut from Teflon tape, 0.015 inch thick, impregnated with 15% glass (Detroit Ball Bearing Co.) were used.

Mineralight, Model 2L 2537 (short-wave ultraviolet).

Rotary evaporator with a thermostatically controlled water bath (45° to 55° C.) and a properly trapped vacuum pump (less than 2 mm.) in combination with glass traps (24/40 adapter, Ace Glass, Inc.).

Beckman Model DU spectrophotometer with a microcell attachment (Beckman 100100 cell sample carrier), microcell holder, and microcells (25 × 3 × 10 mm. internal volume).

Reagents and Solutions. Isonicotinic acid hydrazide (INAH) (Eastman) was prepared daily by dissolving 50 mg. of INAH in 90 ml. of absolute ethanol in a 100-ml. volumetric flask on a mechanical shaker for 30 minutes, adding 1 ml. of hydrochloric acid solution, and making it up to 100 ml. with absolute ethanol.

Nitrogen gas, passed through a Koby filter, for use in evaporating solvents where specified.

Melengestrol acetate standard, 99.5% purity (The Upjohn Co.).

Progesterone (The Upjohn Co.) solution (10 mg. per ml. of absolute ethanol).

Standard, stock solution *A*, 60.0 μg. per ml. (6.00 mg. of melengestrol acetate per 100 ml. of absolute ethanol). Stock solution *B*, 3.0 μg. per ml. (5.0 ml. of stock solution *A* per 100 ml. of ethanol). Stock solution *C*, 6.0 μg. per ml. (10.0 ml. of stock solution *A* per 100 ml. of ethanol). Stock solution *D*, 12.0 μg. per ml. (20.0 ml. of stock solution *A* per 100 ml. of ethanol).

Hydrochloric acid solution (62.5 ml. of concentrated HCl per liter of absolute ethanol).

Solvents. All solvents except two below were distilled in glass or equivalent grade. Anhydrous ethyl ether (Mallinckrodt AR, 1-pound can) was not used if opened more than two hours. Absolute and 95% ethanol (Gold Shield, Commercial Solvents Corp.) must be redistilled if reagent blanks are high.

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PROCEDURE

Preparation of TLC Plates. Prepare 200 × 200 mm. plates using $Al_2O_3GF_{254}$. Place five plates (preferably cleaned by sonic oscillation) in the template, wipe the surface with 95% ethanol, and dry with lintless tissue. Adjust the spreader for 0.75 mm. Weigh out 90 grams of alumina and place in a clean, dry Waring Blendor (two-speed). Add 140 ml. of distilled water and blend at low speed for 30 seconds. After 90 seconds has elapsed from the time the blender was turned on, pour the alumina into the spreader and coat the five plates. Allow the plates to dry a minimum of two days or longer at room temperature. Plates stored up to four weeks are still satisfactory. These conditions may have to be varied from lot to lot to get satisfactory plates.

Activate the plates for 1 hour at 110° C. in an oven and cool 30 minutes in laboratory air (relative humidity 40 to 60%) to dissipate the static charge that builds up during activation. Spot samples and standard as described in the Procedure section.

Carry out the following steps so that the TLC plate will be developed at right angles to the direction in which it is poured. Remove 2 mm. of the alumina from vertical edges of each plate. Scribe the rest of the plate using 2-mm. lines so as to give the following: a TLC plate for fat, muscle, kidney, and tripe—three 54-mm. and one 28-mm. vertical strips—and a TLC plate for liver—two 82 mm. and one 30-mm. vertical strips. Reserve the narrow strip for the standard and wide strips for TLC blank and sample.

Preparation of Sample. Grind the fresh tissue in a meat grinder and store in suitable containers in a deep freeze. Chill the leg bones in the deep freeze for 24 hours, saw them lengthwise (commercial meat band-saw), remove the bone marrow, and freeze. For tripe, steam the rumen wall for 5 minutes and strip off the muscle layer. Store in the deep freeze.

Stopping Places. Samples can be safely stored in the deep freeze after each step provided all solvents are removed.

Extraction Procedure for Muscle, Liver, Kidney, and Tripe. Clean homogenizer by disassembling mixer heads completely and soaking them in detergent with the cups. Keep all parts from each mixer head separated from those of the other assemblies. Brush all parts, and rinse thoroughly with tap water and then distilled water. Let dry and reassemble the mixer heads without using a lubricant.

Weigh 60.0 grams of the thawed tissue into a homogenizing cup. Add 175 ml. of 95% ethanol and a circle of filter paper (Whatman No. 40, 12.5 cm.). Homogenize for 2 minutes in an ice bath, and filter the slurry through filter paper (Whatman No. 40, 12.5 cm., No. 4 Büchner) into a 1-liter filter flask using vacuum. Wash the cup with about 25 ml. of 95% ethanol and filter.

Transfer the filter cake with its filter paper to the cup, and add 175 ml. of 95% ethanol. Again homogenize and filter the slurry. Repeat once more. Mark the level of the combined alcohol eluates, and transfer them to a 2-liter separatory funnel with a Teflon stopcock. Wash the suction flask several times with 95% ethanol, and transfer the washings to the separatory funnel. Then add deionized water to the filter flask up to the level marked on the flask earlier; add 100 ml. more water and 20 ml. of saturated sodium sulfate solution to the flask, mix, and transfer the mixture to the separatory funnel. Add 100 ml. of chloroform, and shake the separatory funnel vigorously for one minute; let stand for 15 minutes or until complete phase separation takes place. If the chloroform layer is less than 50 ml., add 25 ml. more of chloroform and shake again. Drain the chloroform phase into a 1-liter round-bottomed flask. Continue the extraction three more times with 100 ml. of chloroform. Roto-evaporate the combined chloroform extracts, and remove the last trace of water in the following manner: to the contents of the flask, add 25 ml. of (a) 1-to-1 absolute ethanol–hexane and roto-evaporate, (b) absolute ethanol and roto-evaporate, and (c) absolute ethanol and roto-evaporate 15 minutes after solvent has evaporated. Avoid violent bubbling during roto-evaporation.

Extraction Procedure for Fat and Bone Marrow. Weigh 60.0 grams of thawed tissue into a homogenizing cup (see above section). Add 150 ml. of hexane, and warm in a steam bath; stir the solution until the fat dissolves. Do not homogenize the fat, but filter the warm solution through Whatman No. 40 paper (12.5 cm.) into a 1-liter filter flask. Transfer the filter cake, including filter paper, to the cup and add 150 ml. of hexane, then homogenize for two minutes in an ice bath, rewarm, and filter the solution into the suction flask. Repeat the homogenization and extraction of the filter cake one more time. Warm the filter flask and transfer the hexane solution to a 2-liter separatory funnel. Add an additional 500 ml. of hexane, and extract the hexane phase four times with vigorous shaking for one minute with 250 ml. of 90% methanol-water. Each time, drain the lower phase into a second 2-liter separatory funnel. To the combined extracts, add 500 ml. of water and 2 ml. of saturated sodium sulfate solution to give 55 to 60% aqueous methanol. Extract the aqueous methanol with vigorous shaking for 1 minute, once with 300 ml. and three times with 100 ml. of methylene chloride. Combine the extracts in a 1-liter round-bottomed flask, and roto-evaporate as described in the preceding paragraph.

Defatting on Silica Gel Columns. Half-fill a glass column (600 × 28 mm. I.D., with Teflon stopcock and medium porosity sintered glass disks) with hexane, slurry 20 grams of silica gel (50- to 200-mesh, G. Frederick Smith Chemical Co.) in hexane, and pour into the column. Rinse the sides of the beaker and column with hexane. Allow the silica gel to pack by gravity flow of the hexane through the column (8 to 10 ml. per minute). While maintaining at least 15 cm. of solvent, slowly add anhydrous sodium sulfate to a depth of 3 cm. avoiding disruption of the silica gel surface.

Dissolve the residue from the extraction step in 20 ml. of hexane, and transfer it to the column. Adjust the flow rate to about 8 ml. per minute. Wash the flask twice with 10 ml. of hexane, and transfer the washings to the column. Allow the residue to be completely absorbed into the column using additional 10-ml. washes, if necessary, then elute with the following solvents using a small amount of each to rinse the round-bottomed flask, transferring each washing to the column at the appropriate time: 75 ml. of hexane, 350 ml. of 5% ethyl ether–benzene, and 350 ml. of ethyl acetate.

Collect the ethyl acetate fraction in a 1-liter round-bot-

tomed flask and roto-evaporate for 15 minutes after all the solvent has been removed.

Solvent Partition. Transfer the above residue to a 125-ml. separatory funnel (with Teflon stopcock) using each of the following solvents to rinse the round-bottomed flask: twice with 10 ml. of hexane saturated with 70% methanol-water and once with 20 ml. of 70% methanol. Shake the funnel vigorously for one minute, let the phases separate at least one hour, and drain the lower phase into a second funnel containing 20 ml. of hexane saturated with 70% methanol. Shake the second funnel vigorously for one minute, and drain the lower phase into a 500-ml. round-bottomed flask. Repeat shaking of the first and second funnel four more times with 70% methanol (20 ml.), each time washing the round-bottomed flask with the 70% methanol before adding it to the first separatory funnel. Roto-evaporate the combined methanol extracts.

Transfer the residue to a 1-ml. volumetric flask using 2 ml. of 1-to-1 chloroform-methanol. Evaporate the solvents with a stream of nitrogen, and repeat the transfer and evaporation two more times. Then add 10 drops of 1-to-1 chloroform-methanol to the 1-ml. flask and once again evaporate.

Thin-Layer Chromatography. To the residue in the 1-ml. volumetric flask, add 10 drops of 1-to-1 chloroform-methanol. Transfer the entire 10 drops to a freshly prepared plate using a 25-μl. disposable pipet (Microcap, Drummond Scientific Co.). Apply the sample 2 cm. from the bottom of one of the strips. Rinse the flask with 5 drops of 1-to-1 chloroform-methanol and again apply to the plate. Repeat once more. Leave one strip blank to serve as a TLC blank. Then spot 5 μl. of melengestrol acetate standard (1 mg. per ml.) and 5 μl. of the progesterone (1 mg. per ml.) on top of each other on the narrow strip.

Allow the plate to dry for 10 minutes at room temperature and for 10 additional minutes under vacuum at room temperature. Then place it in a tank containing fresh 1-to-1 chloroform-methanol. Remove the plate from the tank when the solvent front has moved 5 cm. from the bottom of the plate. Dry for $^1/_2$ hour at room temperature, again drying the last 15 minutes under vacuum. Finally, place it in the fresh 10:1:1 benzene–chloroform–ethyl acetate, and allow the solvent to rise 17 cm. from the bottom of the plate. Remove the plate and dry on a cork ring in a horizontal position so that it does not touch the bench top.

Determine the position of melengestrol acetate on the plate with ultraviolet radiation. For low-level samples, use the reference standard as a guide; melengestrol acetate will be below progesterone. Scribe horizontal lines on the plate above and below the melengestrol acetate spot. Avoid the progesterone area. Remove the melengestrol acetate zone with a razor blade, and transfer the scrapings to a sintered glass funnel (fine porosity, 2.5 × 5.0 cm.). Also remove a similar zone from the strip designated as the TLC blank. Wash the funnels and contents four times with 5 ml. of 19-to-1 ethanol (prepared from absolute ethanol and distilled water), each time stir with a glass rod, let stand 5 minutes and filter with vacuum into a 50-ml. round-bottomed flask. Roto-evaporate the combined filtrates.

Spectrophotometric Determination. To the dry residue in the 50-ml. round-bottomed flasks (samples and standards), add 1 ml. of isonicotinic acid hydrazide (INAH) reagent in groups of three, 10 minutes apart. Stopper, swirl, allow to stand for 15 minutes, and swirl again. After 1.0 hour, measure the absorbance of samples and standards (in groups of three) at 415 mμ.

Standard Curve. Prepare a standard curve (1.5, 3, 4.5, 6, 9, and 12 μg. per ml.) by pipetting the appropriate volume of stock solutions *B*, *C*, and *D* into a 50-ml. round-bottomed flask and roto-evaporate. Add 1 ml. of the color reagent and proceed as described above.

Calculations. Plot the micrograms of melengestrol acetate found against the absorbance (*A*). Determine the slope and calculate the amount of melengestrol acetate from the following formulas (for a 60-gram sample).

$$\text{P.P.B.} = \frac{\mu\text{g. of MGA (standard)}}{A\text{ (standard)}} \times \frac{1000}{60} \times [A\text{ (sample)} - A\text{ (TLC blank)}]$$

RESULTS AND DISCUSSION

Recent literature indicated that most of the methods for the detection of natural progestogens in body tissues (placental, for example) were developed to find finite amounts —i.e., 1 to 10 p.p.m. (Nienstedt, 1967)—in which the sensitivity of the method was not critical to the related studies. This is to be contrasted with this residue procedure in which the sensitivity was paramount and was directly related to sample size and the specific response of the end point measurement necessitating use of the entire sample in the final measurement. During the preliminary stages of development, it became evident that 50 p.p.b. was easily detectable and that under conditions in which the background contribution and its variability were known, 25 p.p.b. could be detected.

Sensitivity of the Method. For the melengestrol acetate residue studies, the sensitivity of the method was determined by calculating the variability of the assay value of control tissue from untreated animals about their group mean. A significant value (positive MGA residue) was indicated for a tissue of a treated animal when it exceeded the average of the control value by three standard deviations. A summary of background tissue residue results from all control animals assayed to date and their values of significance have been calculated for each tissue (Table I). The average standard deviation for the five

Table I. Summary of Observed Background Tissue Residue Values for Melengestrol Acetate in Untreated Heifers

Tissue	No. of Animals	Values in P.P.B. MGA: Average	Std. dev.	Significance for a single animal
Fat	25	12.6	±8.9	39.3
Liver	21	10.5	±8.5	36.0
Muscle	20	7.1	±4.2	19.7
Kidney	21	7.2	±7.1	28.5
Bone marrow	21	10.8	±8.4	36.0

tissues was 7.4 p.p.b., thus the sensitivity of the method has been demonstrated to be any value greater than three standard deviations—22.2 p.p.b. (approximately 25 p.p.b.)—above control values. Since these values are for a single animal, the values are maximal. For a group average, which was usually the case in residue work, the values of significance were lower.

Recovery Studies. Recovery studies were based on the amount of melengestrol acetate found after fortification of homogeneous tissue samples with a known amount of the steroid. For each tissue, aliquots of the unfortified sample were analyzed to determine the contribution made by the tissue per se. An example of a typical recovery study (Table II) shows the actual range of absorbance values obtained and the p.p.b. found for three fortified and unfortified tissues. The low absorbance values obtained for the TLC blank, control tissue, and the tissue fortified with 25 p.p.b. of MGA indicated that the method has been pushed to its practical limit because of the variability of the thin-layer plate blanks and that some tissues, particularly liver, the most difficult tissue to clean up, produced significant background levels. Despite these factors, a comparison of the averages of fortified and unfortified values indicated that satisfactory recoveries ranging from 72.6 to 97.2% could be obtained at the 25-p.p.b. level. Wide variation in recovery is expected to occur at the limit of sensitivity of any method. Fortification at the 50 to 200 p.p.b. level (Table III) showed the average recovery to be 81.2% with a standard deviation of ± 10.1%. These data show that higher levels of MGA can be detected in these tissues with no difficulty when the sensitivity of the method is exceeded.

A Typical Residue Study. Each residue study included a group of untreated animals so that a statistical comparison of the results from treated and untreated animals could be made (Table IV). Application of student's t test indicated no significant difference between the untreated and treated group which had been withdrawn from treatment two days prior to slaughter.

A Summary of MGA Residue Studies. Numerous studies were conducted to determine the withdrawal time for MGA-treated heifers (Table V). Among those was an overdosage study to show which tissue had a preference for this steroid. One beef heifer was fed 1000 mg. per day for 5 days; on the fifth day 500 mg. subcutaneously and 500 mg. intramuscularly were given, and on the sixth day the animal was slaughtered. Assays indicated 3300 p.p.b. in fat, 2100 p.p.b. in bone marrow, 880 p.p.b. in liver, 220 p.p.b. in muscle, and 120 p.p.b. in kidney. This study indicated that fat should be the first tissue monitored to establish possible MGA levels.

Although all tissues, fat, muscle, liver, kidney, bone marrow, and tripe were assayed, only a summary of all fat tissue assays from treated animals is presented to show safe withdrawal time (Table V). At elevated dosage levels (1.6 mg. daily for 182 days), detectable amounts of steroid were observed in the fat 1.5 days following treatment (see Table I for minimum values). At the recommended dosage level of 0.4 mg. daily for 182 days, a detectable amount was found in fat 12 hours post-treatment. However, in another group fed 0.6 mg. daily for 101 days (Table V), no detectable tissue levels were observed when animals were slaughtered following no withdrawal period from treatment. Differences in feedlot experimental conditions may be the

Table II. Recovery of Melengestrol Acetate from Ground Fat, Liver, and Muscle Tissue Fortified at 25 P.P.B.[a]

	Absorbance[b]			P.P.B. MGA			
Tissue[c]	Sample	TLC blank	Sample less blank	Found	Average	Net recovery	% recovery
Unfortified fat	0.032	0.011	0.021	7.7			
	0.031	0.015	0.016	5.8			
	0.029	0.015	0.014	5.1	6.2		
Fortified at 25 p.p.b.							
	0.081	0.011	0.070	25.6			
	0.084	0.015	0.069	25.2			
	0.084	0.015	0.069	25.2	25.3	19.1	76.4
Unfortified liver	0.104	0.018	0.086	30.1			
	0.066	0.022	0.044	15.8			
	0.069	0.017	0.052	18.7	21.5		
Fortified at 25 p.p.b.							
	0.112	0.009	0.103	37.1			
	0.160	0.024	0.136	48.1			
	0.170	0.025	0.146	52.2	45.8	24.3	97.2
Unfortified muscle	0.031	0.009	0.022	8.0			
	0.037	0.013	0.024	8.8	8.4		
Fortified at 25 p.p.b.							
	0.093	0.014	0.079	28.8			
	0.085	0.009	0.076	27.7			
	0.091	0.013	0.078	28.5	28.3	19.9	72.6

[a] Sample size, 60 grams of ground tissue. Fortification level, 1.5 μg. in 1 ml. of absolute ethanol.
[b] Absorbance values were measured in a DU spectrophotometer using microcells having a 1-cm. light path.
[c] Each tissue was taken from the same lot.

Table III. Recovery of Melengestrol Acetate from Ground Fat, Liver, and Muscle Tissue Fortified at 50, 100, and 200 P.P.B.[a]

Tissue	P.P.B. MGA Added	P.P.B. MGA Found	P.P.B. MGA Net[b]	% Recovery[c]
Fat	0	9.2		
	0	4.2 } 6.1[b]		
	0	5.0		
	50	53.3	47.2	94.4
	50	44.2	38.1	76.2
	50	40.8	34.7	69.4
	100	83.3	77.2	77.2
	100	83.3	77.2	77.2
	100	96.7	90.6	90.6
	200	161.7	155.6	77.8
	200	177.5	171.4	85.7
	200	167.5	161.4	80.7
Liver	0	16.7		
	0	21.7 } 26.1[b]		
	0	40.0		
	50	49.2	23.1	46.2
	50	65.0	38.9	77.8
	50	65.0	38.9	77.8
	100	118.3	92.2	92.2
	100	118.3	92.2	92.2
	100	119.2	93.1	93.1
	200	185.8	159.7	79.9
	200	184.2	158.1	79.1
	200	190.0	163.9	82.0
Muscle	0	9.2		
	0	16.7 } 14.5[b]		
	0	17.5		
	50	59.2	44.7	89.4
	50	56.6	42.1	84.2
	50	61.7	47.2	94.4
	100	91.7	77.2	77.2
	100	95.0	80.5	80.5
	100	88.3	73.8	73.8
	200	188.3	173.8	86.9
	200	187.5	173.0	86.5
	200	154.2	139.7	69.9

[a] Sample size, 60 grams ground tissue. Fortification level, 3.0, 6.0, and 12.0 μg. in 1 ml. ethanol.
[b] Three sample averages of the controls used to calculate net values.
[c] For fat, liver, and muscle the pooled average is 81.2 ± 10.1%.

Table IV. Typical Data for Melengestrol Acetate Assay in Beef Heifer Tissue Following Two-Day Withdrawal Period[a]

Days Post-Treatment	Number	Apparent P.P.B. MGA Fat	Liver	Muscle[b]	Bone marrow	Kidney	Rumen wall, tripe
Control	68	18.3	32.2	14.8	11.7	0.4	4.9
Control	152	5.8	2.4	14.4	7.1	16.0	23.7
Control	353	32.1	20.0	6.8	7.1	8.2	42.8
Control	35	1.7	19.6	6.2	7.1	9.2	16.2
Control	142	2.1	9.6	6.6	0.8	15.5	6.5
Control	271	6.2	13.3	7.2	24.2	32.6	3.6
Average		11.0	16.2	9.3	9.7	13.6	16.3
Standard deviation		±12.0	±10.2	±4.1	±7.9	±10.9	±15.1
2 days	18	9.6	7.2	8.4	9.2	4.5	14.3
2 days	94	13.7	2.0	26.0	12.5	13.6	16.3
2 days	177	12.5	0.8	8.0	32.9	7.4	8.2
2 days	11	19.6	13.3	8.8	0.0	5.0	21.0
2 days	149	13.7	13.7	11.8	15.4	2.1	21.9
2 days	120	15.0	9.6	9.7	15.4	15.1	17.8
Average		14.0	7.8	12.1	14.2	8.0	16.6
Standard deviation		±3.3	±5.5	±6.9	±10.8	±5.3	±5.0
t value[c]		0.59	−1.77	0.85	0.83	−1.15	0.05

[a] Treated animals fed 0.4 mg. per day for 194 days and slaughtered two days post-treatment. Tissues taken at time of slaughter, ground and frozen.
[b] Last three values in control and treated heifers are average of duplicates.
[c] Analyzed by t test for unpaired values, 10 degrees of freedom (t 0.01 = 3.17) (t 0.05 = 2.23). All t values indicated no significant difference.

Table V. A Summary of MGA Assays in Fat Taken from Heifers Fed Various Levels of Melengestrol Acetate and Slaughtered at Different Withdrawal Times[a]

Treatment		No. of	Average P.P.B. MGA Obtained after Various Days Post-Treatment[b]					
Dose, mg.	Days	Animals	0	1/2	1	1 1/2	2	4
0.85	111	1			18			
0.6	185	6					13	
0.6	101	6	7				8	5
0.53	198	1			15			12
0.4	279	6					9	
0.4	194	6					14	8
0.4	185	6					10	
1.0	236	3					21	
1000.	5[c]	1	3300					
1.6	182	2		108		76		
0.4–1.6[d]	182	2		52		46		
0.4	182	6		32				

[a] Fat was taken from the kidney knobs (perirenal fat).
[b] Only values in the lower part of the table (below the line) are considered detectable or positive.
[c] On the fifth day the heifer was given 500 mg. subcutaneously and 500 mg. intramuscularly.
[d] Fed 0.4 mg. per day for 56 days followed by 1.6 mg. for 126 days.

factor causing this type of variation. All values in this group (upper half of Table V) were negative. Based on these data, a two-day withdrawal period appears adequate for steroid clearance from tissues even with doses up to 1.0 mg. daily. This rapid clearance is consistent even for long treatment periods (279 days) as well as shorter periods (101 days).

This table shows that the method is adequate for establishing withdrawal times for heifers fed melengestrol acetate so that tissue residues do not exceed the 25-p.p.b. level.

Progesterone Assay. During routine fat assay, the ultraviolet absorption pattern of thin-layer chromatograms for control and treated heifers differed. In all cases, control fat was found to have an additional dark band 1 cm. above melengestrol acetate. Based on infrared absorption, mass spectroscopy, GLC retention time, and R_f on alumina, this band was identified as 4-pregnene-3, 20-dione (progesterone). As a result, both melengestrol acetate and progesterone were routinely spotted on TLC plates to assure that complete separation of these two steroids had occurred in each day's run.

This distinct separation makes this method useful for progesterone assay, provided the INAH reaction mixture is read at 275 mμ. No difficulties were encountered in over 76 progesterone assays in the 50- to 500-p.p.b. range.

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