Measurement and Presence of Melengestrol Acetate (MGA) in Beef Tissues at Low Levels

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A screening and confirmative method for measuring the steroidal hormone melengestrol acetate (MGA) in beef tissues at low levels has been developed. Beef fat was extracted with hexane and beef tissues with acetonitrile and the residues partitioned between hexane and methanol–water and then extracted into chloroform. The MGA in the sample was then qualitatively analyzed by GLC enabling the presence of 2 ppb to be detected in a 50-g sample. Confirmation and quantitation were effected by further purification on a Florisil column followed by GLC analysis. The MGA was then hydrolyzed in base to melengestrol and GLC properties of this alcohol were used to verify the mother compound. Beef tissues and fat from heifers fed 0.4 mg per day of MGA were analyzed after a 48-hr withdrawal period. Levels of 5–10 ppb were found in fat, 1–5 ppb in liver, and lesser amounts in muscle and kidney.

Melengestrol acetate (MGA) is a powerful progestational steroid used to finish beef heifers which are intended for the food market and not for calf production. The effect of this hormonal feed additive on the animal is to inhibit ovulation and suppress estrus resulting in easier management in a feedlot environment. Concomitant with this effect is an increased weight gain of the heifers (up to 11%) when compared to nonmedicated ones and also an improvement (up to 8%) in conversion of feed to meat (Zimbelman et al., 1970). The dosage used in feed is calculated to give 0.25–0.50 mg per animal per day and all medicated feed must be withdrawn 48 hr before slaughter. Because of the strong physiological effect of this hormone, similar to that of contraceptive hormones, residues of this compound in beef tissues must be low. For this reason and because of consumer concern about any animal feed additive appearing in human food, it is desirous to have sensitive reliable methods available for measuring MGA in beef tissues.

Techniques have been developed for measuring MGA in animal feeds (Davis et al., 1972) and bovine tissue (Krzeminski and Cox, 1973). Due to the low residue levels expected and lack of agreement as to what level of this animal hormone is a health concern, we believed it was necessary to have a more sensitive (1–2 ppb rather than 25 ppb) and shorter procedure than that developed by Krzeminski and Cox (1973). It was also thought desirable to have confirmative techniques available such that more than the appearance of a gas–liquid chromatographic (GLC) peak for MGA was required to designate a food tissue as containing this chemical. To this end, we used the extraction procedure of Krzeminski and Cox but developed new purification and confirmative procedures for MGA. This paper describes a relatively simple procedure for screening MGA residues in beef tissue down to 2 ppb along with additional steps to be used for confirmation and quantitation of positive samples. This method so developed is then used to measure the presence of MGA in tissues from animals fed this hormone and subjected to a withdrawal period of 2 days.

EXPERIMENTAL SECTION

Apparatus. (a) The gas chromatograph used was a Hewlett-Packard 5700 or equivalent equipped with a Ni-63 high-temperature electron capture detector, 1-mV recorder, and all-glass on-column injection system. (b) The blender used consisted of jars fitted with gaskets (Teflon or otherwise) which give no interfering GLC peaks when in contact with organic solvents.

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Reagents. (a) Solvents used were glass distilled residue grade which give minimal response to electron capture when concentrated to dryness. (b) Melengestrol acetate was an Upjohn control reference standard available from Tuco Products, Orangeville, Ontario, Canada. Solutions were: (i) stock solution; 5.0 mg of standard MGA was added to a 50-ml volumetric flask and dissolved in acetone; (ii) spiking and GLC solution; 0.5 ml of the above stock solution was added to a 50-ml volumetric flask and made to volume with hexane. (c) Celite 545 was acid washed (Filter Aid) from Johns Manville. (d) Florisil (Applied Science Lab., Inc., Pesticide Grade) was activated at 300° overnite and 2% distilled water was added. The mixture was shaken for 2 hr on a mechanical shaker and stored in a desiccator. (e) Column packing used included 1% OV-17 and 1% OV-210 both on Gas-Chrom Q, 80–100 mesh or the equivalent (Applied Science Laboratories, Inc.).

Preparation of Samples. The entire portion of tissue and fat was ground in a meat grinder, divided into 50-g aliquots in plastic bags, and stored in a freezer at −10°.

Extraction Step. (a) *Fat.* The procedure of Krzeminski and Cox (1973) was used with the following modifications. The Celite on filter flask was prewashed with 2 × 100 ml portions of acetonitrile (CH_3CN) to remove impurities which show up on the GLC chromatogram. After filtration of the sample, the beaker, blender, and Celite were not rinsed with CH_3CN.

(b) *Tissue (Muscle, Liver, Kidney).* Fifty-gram samples were placed in the blender jar, containing 50 g of anhydrous granular Na_2SO_4 and 150 ml of acetonitrile, and the mixture was homogenized for 3 min. At the same time 20 g of Celite was placed in a sintered glass funnel and washed with 2 × 100 ml portions of CH_3CN and dried with suction (discarding the CH_3CN). The homogenate was filtered through the Celite cake under vacuum and the blender jar was rinsed with a little CH_3CN. The top portion of the Celite cake containing the tissue was transferred with a spatula to the blender jar which contains 25 g of anhydrous Na_2SO_4 and 150 ml of CH_3CN, and the mixture was homogenized and filtered as described previously. The combined CH_3CN extracts were then shaken with 3 × 100 ml portions of hexane in a 1-l. separatory funnel (which were discarded) and transferred to a 500-ml flask containing 5 g of powdered anhydrous $MgSO_4$. After 5 min, the mixture was filtered through paper into a 1-l. round-bottomed flask, 50 ml of benzene was added, and the contents evaporated to dryness on a rotary evaporator at 25–35° under vacuum.

Partition Step. The residue from either fat or tissue was triturated with 3 × 15 ml portions of hexane saturated with methanol–water–Na_2SO_4 (70:30:0.1, v/v/w), S_1, and trans-

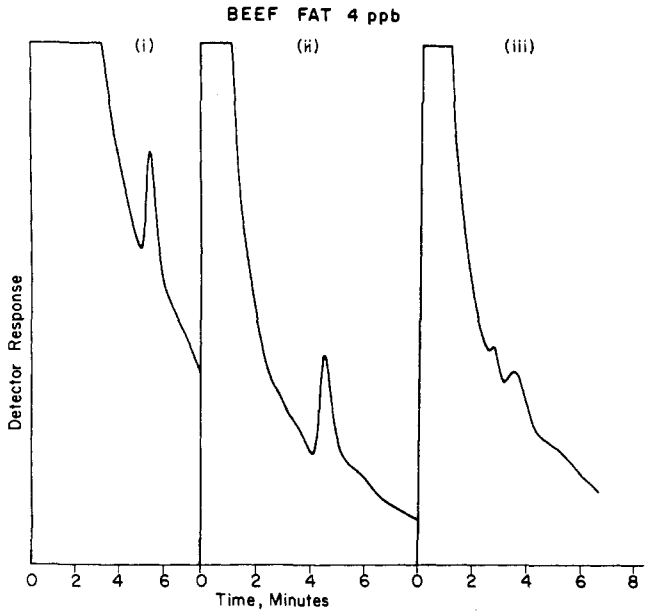

Figure 1. GLC tracings on OV-17 of beef fat containing 4 ppb of MGA: (i) after extraction and partition; (ii) after Florisil; (iii) after hydrolysis and Florisil.

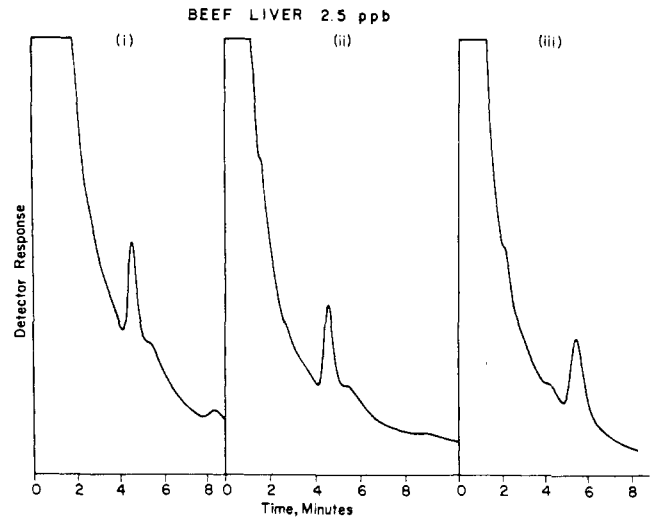

Figure 2. *GLC tracings of beef liver containing 2.5 ppb of MGA: (i) after extraction and partition, OV-17; (ii) after Florisil, OV-17; (iii) after Florisil, OV-210.*

ferred to a 250-ml separatory funnel. The S_1 solvent was extracted with methanol–water–Na_2SO_4 (70:30:0.1) saturated with hexane, S_2 solvent, once with 25 ml and twice with 15-ml portions. (*Caution:* emulsions can form at this point necessitating a gentle contact of two phases.) The combined S_2 was back washed with a 1 × 10 ml portion of S_1, 10 ml of water was added, and MGA was extracted with 3 × 20 ml aliquots of $CHCl_3$. The $CHCl_3$ washings were dried over anhydrous powdered $MgSO_4$ (1 g) for 15 min, filtered through paper into a 250-ml round-bottomed flask, and evaporated to dryness at 25–35° under vacuum. The residue was transferred to a 50-ml round-bottomed flask with three small aliquots of hexane and evaporated to dryness and 1 ml of hexane was added. The solution was allowed to stand at room temperature for 15 min to allow precipitation of impurities and then analyzed qualitatively by GLC.

Gas Chromatography. Operating conditions were: 2.5–3 ft long × 4 mm i.d. all-glass columns packed with either 1% OV-17 or 1% OV-210 both on Gas-Chrom Q, 80–100 mesh; detector, 300°; injection port, 250°; oven, with OV-17, 260°; with OV-210, 235°; carrier gas, argon–methane (5 to 20%), 60 ml/min flow. At attenuation 16 2–5 μl of 1 ml of solution was injected. With these conditions retention time for MGA was between 4 and 6 min. It was also found that the peak sensitivity for MGA was increased by first injecting a hexane solution of beef fat onto the column, allowing the pen to return near zero, and then injecting the standard. If no MGA peak was observed after extraction and partition, the sample was not examined further.

Confirmation by Florisil Chromatography. A 2-g slurry of activated Florisil in hexane was added to a 10-mm i.d. glass column equipped with a Teflon stopcock. The adsorbant was washed successively with 50-ml portions of acetone and hexane. The hexane solution of the sample was transferred to the column which was washed with 25 ml of hexane–ethyl acetate (9:1). The MGA was eluted with 25 ml of hexane–acetone (1:1) and the eluent evaporated to dryness and taken up in 1 ml of hexane. The solution was then analyzed on OV-210 by itself and by peak enhancement when coinjected with a standard. A standard curve of peak height vs. concentration in the range 0.2–1.0 ng was made up on OV-210 and the peak height of sample compared to the value on the standard curve. From this value, the amount in parts per billion in the entire sample was then calculated.

Confirmation by Hydrolysis and GLC. The hexane solution of the sample after Florisil cleanup was taken to dryness, 3 ml of ethanol was added to redissolve the sample, 2 ml of ethanolic 0.5 *N* KOH was added, and solution was kept at room temperature for 90 min. Ten milliliters of 0.25 *N* HCl and 5 ml of water were added and the mixture was extracted with 3 × 10 ml portions of $CHCl_3$. The $CHCl_3$ extract was washed with 2 × 10 ml aliquots of H_2O, dried with a little anhydrous $MgSO_4$, filtered through paper, evaporated to dryness, and taken up in 1 ml of hexane. The sample was then analyzed by GLC. The melengestrol (MG) peak appeared at about 1 min shorter retention time than MGA with the peak for MG broader than MGA. At levels below 4–5 ppb as determined by GLC after Florisil, the hydrolyzed sample containing the MG must be purified with a second Florisil column in order for the MG peak to be distinguished from background.

Before beef samples were analyzed, a solvent fortified with MGA at the 0-, 2-, and 5-ppb levels, and a homogenized tissue sample fortified at the same levels, should be processed through the entire method to ensure the purity of reagents, amount of interference from tissue, and the percent recovery.

RESULTS AND DISCUSSION

Method. A convenient property of MGA is its elution on a GLC column without derivatization and the small quantities (less than 1 ng) that can be readily detected using its electron capturing properties. Because of the instability of MGA at high temperatures, the columns must be short, all glass with on-column injection, and a light loading of stationary phase used. Using OV-17 at 260°, the MGA peak is somewhat skewed with a tailing peak; at 270°, breakdown occurs. Using OV-210 at 235°, the peak shape is symmetrical.

A previous method by Krzeminski and Cox (1973) for MGA was sensitive to 25 ppb at which level no MGA could be detected in heifers on a 0.4 mg per day dosage after a 2-day withdrawal, the so-called "zero tolerance". This procedure involved an extraction step, purification by chromatography on alumina followed by measurement on GLC using electron capture. Since a lower level of detection was desired to measure the actual amount of MGA present, and because problems were encountered by us with the alumina chromatography, the cleanup was modified and shortened to give less GLC interference and greater sensitivity.

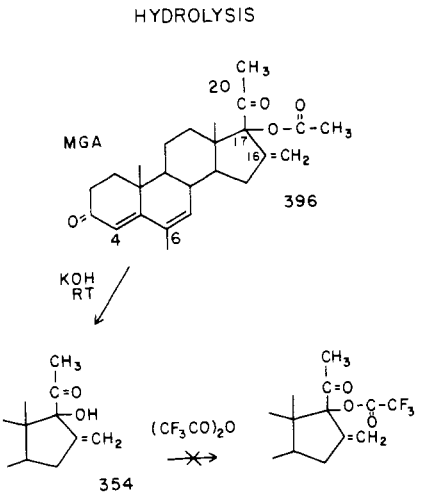

Figure 3. Hydrolysis of MGA.

To this end, the residue after extraction was partitioned between hexane and methanol–water. More water was added and the MGA extracted into chloroform. Further purification of tissue samples (except fat) was also effected by dissolving the final extract in hexane, and allowing impurities to precipitate out at room temperature before GLC analysis. This relatively simple procedure yielded an extract which was suitable for GLC–electron capture and could readily screen qualitatively for MGA down to a level of 2 ppb without interference from extraneous peaks. Tailing of the initial peak was somewhat of a problem and, at low levels of 2–5 ppb, the MGA was present on a sloping base line as shown in Figures 1 and 2. If no MGA is observed at the 2-ppb level after partition and GLC, the sample is not examined further.

Confirmation by Florisil and GLC. Those samples which, after extraction and partition (E–P), appeared to be positive for MGA were adsorbed on a small amount of Florisil, the MGA eluted with hexane–acetone, and analyzed on GLC using OV-210. As the background caused by extraneous material was now much reduced, larger amounts could be injected on the GLC at the same attenuation resulting in ease of quantitation using peak heights. Using the OV-210 phase, the sample was analyzed at 235° (260° for OV-17) and peak retention time, peak enhancement, and quantitation affected.

Confirmation by Hydrolysis and GLC. As a final confirmatory step, the sample containing MGA was hydrolyzed (Figure 3) in mild base to yield melengestrol (MG) and GLC analysis was again performed. Since the MG peak was not as well resolved on GLC, at low levels (below 5 ppb as determined after the first Florisil cleanup) the sample after hydrolysis was passed through a second Florisil column to separate MG from background interference. This new compound produced on basic hydrolysis of MGA was characterized by its mass spectrum and is believed to be the alcohol of MGA, i.e. melengestrol. It was not possible for us to obtain a molecular ion peak for MGA after GLC nor to convert MG to a fluoroacetate. GLC patterns of all these samples and their changes on further purification are shown for beef fat at 4 ppb (Figure 1) and for beef liver at 2.5 ppb (Figure 2). In particular, the improvement of solvent tailing on treatment of the sample with Florisil and the shape of the MG peak are evident.

Beef tissues were obtained from local markets and analyzed and were found to contain no MGA. These control samples were subsequently fortified at several levels with MGA and recoveries are shown in Table I. The losses of MGA are not due to its deterioration with time as this steroid is stable indefinitely at room temperature. However, in these experiments we have concentrated on the facility of their performance particularly for a qualitative screening technique and this has resulted in some loss of material, e.g. the residue after evaporation of acetonitrile is extracted only with hexane before partitioning. This results in a type of cleanup and improved GLC pattern but a small amount of MGA is trapped in oily residue and lost.

The sensitivity of this method is not easy to define. There is virtually no background-electronic noise on the recorder at working levels (attenuation 16) used since the amount appearing is only the width of the pen line (~0.5 mm). The difficulty then becomes to distinguish the MGA peak in a sample from the sloping-background tailing of solvent and extraneous material. We have used a value for peak height of MGA of 10 mm as our minimum detectable amount. With this criterion a level of 2 ppb (0.5 ng from a 5-μl injection in a 1-ml solution) can be detected qualitatively in beef fat after only E–P and this can be confirmed and quantitated by Florisil chromatography, hydrolysis, and GLC. If a sample is not positive after E–P, it is not examined further. Detection can be increased to 0.5 ppb on OV-210 if all three steps are used. For tissue samples, these values are 1 ppb after extraction and partition and 0.5 ppb after Florisil cleanup.

No published study has appeared on the metabolism or conjugation of MGA in beef cattle although the fate in humans and rabbits has been studied by Cooper et al. (1967). In women, most of the MGA is excreted either intact or as the 2-hydroxy glucuronide and in rabbits the steroid is excreted intact along with some C_6 methylhydroxy glucuronide. No hydrolysis step was included in the extraction procedure since MGA cannot conjugate without first being metabolized and, in beef cattle, the metabolic changes have not been defined.

Table I. Recovery of MGA from Fortified Beef Tissues

Tissue	Level of fortification, ppb	% recovery[a]
Fat	10	74
	5	80
	2	56
Liver	5	81
	2	55
	1	58
Muscle	5	63
	2	59
	1	56

[a] Average of at least two samples after extraction, partition, and Florisil. Analysis by GLC on OV-210.

Table II. MGA Content[a] of Tissues from Medicated Heifers in ppb

	Tissue[a]				
Animal[b]	Fat	Perirenal fat	Muscle	Liver	Kidney
1	9.4	4.7	1.8	4.2	tr[c]
2	5.6	2.5	1.1	1.5	tr
3	5.9	3.1	1.5	1.5	tr
4	5.2	2.2	1.8	1.4	tr
5	4.5	1.2	0.7	1.1	tr
6	5.6	4.0	0.9	1.1	tr
7	6.5	4.8	1.5	0.9	1.0
Av	6.1	3.2	1.3	1.7	

[a] Corrected for recovery (Table I). [b] Animals 1 and 2 were from two different farms in Alberta and animals 3–7 were from the same farm in Ontario. [c] Traces, between 0.2 and 0.5 ppb.

Presence in Beef Tissues. Tissues and fat were obtained from seven different 16–18-month-old beef heifers from three farms in two provinces. These animals were in a feedlot environment for finishing and were fed MGA-100 (Tuco) (ca. 0.4 mg/animal per day) for at least 2 months. Feed was withdrawn 48 hr before slaughter and tissue (muscle, liver, kidney, subcutaneous fat, and perirenal fat) was obtained and stored at −10°F until analyzed (up to 2 months as MGA is stable in storage). The MGA contents of these tissues using the described method are shown in Table II. The largest amount of MGA is in the fat (5–10 ppb) and this agrees with earlier work of Krzeminski et al. (1968) who found this tissue to contain most of this lipid-soluble steroid. Lesser amounts are found in the liver (1–5 ppb) and muscle with only traces in the kidney. At present, a zero tolerance has been established for MGA residues in foods, a tolerance which depends on the method sensitivity which, in turn, invariably increases with the advance of techniques. The residue values of MGA reported here in beef tissue combined with toxicological data should form a basis for a tolerance in food and a withdrawal period for this drug in animal practice.

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An Immobilized Glucose Isomerase for the Continuous Conversion of Glucose to Fructose

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The conversion of glucose to fructose in column reactors, by glucose isomerase adsorbed on the internal surface of controlled-pore alumina, has been investigated. All column reactors were evaluated with substrate solutions containing either 36 or 50 g of glucose per 100 ml of solution at 60°. Magnesium and cobalt ions were included in the feed. Sodium sulfite was employed to regulate the substrate pH. Initial column studies were performed with adsorbed enzyme derivatives having activities in the range of 200–400 glucose isomerase units (GIU) per gram of material, as determined by a static assay procedure. Column half-lives in excess of 40 days were observed for reactors operating at 80 to 85% theoretical conversion of glucose to fructose. Subsequent column reactors containing derivative with activities between 550 and 650 GIU/g of material, as measured by actual column operating parameters, also showed half-lives greater than 40 days.

The enzymatic conversion of glucose to fructose, via an isomerase extracted from *Pseudomonas hydrophila* cells grown on xylose, was reported by Marshall and Kooi (1957). Initial studies on glucose isomerase obtained from *Streptomyces* sp. were reported by Tsumura and Sato (1965). These authors demonstrated a dependency of the *Streptomyces* isomerase upon cobalt and magnesium ions for activation, and showed that cobalt ion protects the enzyme from high temperature denaturation. Takasaki (1966) and Takasaki et al. (1969) further detailed the effect of metal ions, temperature, and pH on the *Streptomyces* isomerase performance.

The glucose isomerase obtained from the *Streptomyces* organism is an endocellular enzyme, and is therefore costly to obtain because of the processing required to liberate it from cellular material. Since the sugar industry may be considered producers of commodity products, high cost enzymes must be employed in the most economical manner. Soluble enzymes are used in but one batch reaction; however, if the enzyme is immobilized, it not only can be used many times, but can also be employed in continuous reactors.

Sugar, sucrose, is at the present time in short supply. The price to the consumer has been increased from 200 to 300% during the past year. An alternative supply of a natural sweetener would relieve, to some degree, the demand for sugar and thus reduce the price to consumer. *Streptomyces* glucose isomerase can convert 50% of the glucose present in solution to fructose. This 50% glucose–50% fructose solution may be used as a substitute for invert sugar in products marketed by the soft-drink beverage and confectionary industry. Therefore, glucose isomerase immobilized by an economical procedure and judiciously employed in the conversion process is of considerable interest for such applications. This report describes the performance advantages and limitations of columns containing *Streptomyces* isomerase, adsorbed on the internal surface of controlled-pore alumina.

EXPERIMENTAL SECTION

Materials. The glucose isomerase utilized for these studies was derived from a *Streptomyces* sp. The activity of the various native enzyme preparations ranged from 270 to 450 glucose isomerase units (GIU)/g.

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