MELENGESTROL ACETATE

First draft prepared by

Adriana Fernández Suárez, Buenos Aires, Argentina

Dieter Arnold, Berlin, Germany

ADDENDUM

To the monograph prepared by the 54th meeting of the Committee

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IDENTITY

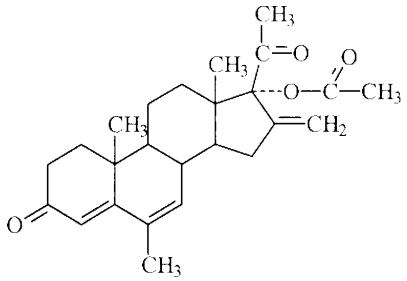
Chemical names: 17α-Acetoxy-6-methyl-16-methylenepregna-4,6-diene-3,20-dioneacetate (Chemical Abstract name)

Melengestrol Acetate (International Non-Proprietary Name)

CAS-No: 2919-66-6.

Manufacturer's code: PNU-21240

Structural formula:



Molecular Formula: $C_{25}H_{32}O_4$

Molecular weight: 396.53

OTHER INFORMATION ON IDENTITY AND PROPERTIES

Appearance: Off white-to-light yellow powder.

Melting point: 224-226°C

Optical rotation: $[\alpha]_D^{23}$ -127° (c=0.31 in chloroform)

Ultraviolet maximum: 287 nm (in ethanol; log ε 4.35)

Solubility: 1.06 mg/L in water

INTRODUCTION

Melengestrol acetate (MGA) is a orally active progestational steroid used to improve feed conversion efficiency, promote growth, and suppress oestrus in beef heifers. The range of approved doses is 0.25 to 0.50 mg/heifer/day. Melengestrol acetate is fed for the duration of fattening/finishing period, usually 90 to 150 days.

Melengestrol acetate has been previously evaluated at the 54th meeting of the Committee, and temporary maximum residue limits (MRLs) were recommended. These temporary MRLs were 5 µg/kg for bovine fat and 2 µg/kg for bovine liver.

In reaching its decision on MRLs, the Committee took into consideration the following factors:

- An ADI of 0-0.03 µg/kg of body weight was established, which is a equivalent to a maximum ADI of 1.8 µg for a 60-kg person.
- Only fat and liver contain concentrations of the marker residue that are quantifiable on a routine basis; methods with limits of quantification greater than 0.3 µg/kg are unlikely to permit quantification of residues in muscles from animals treated with recommended doses of melengestrol acetate.
- The sponsors did not submit an analytical method suitable for monitoring purposes.

Therefore, the Committee recommended temporary MRLs and requested the following information:

- A analytical method suitable for quantifying residues of melengestrol acetate in liver and fat tissues.

In response to the Committee's request, the sponsor provided a validated chemical method for evaluation by the 58th JECFA meeting.

METHODS OF ANALYSIS FOR RESIDUES IN TISSUES

A validated HPLC-MS method for the determination of melengestrol acetate residues in bovine fat and liver tissues was submitted.

Quality assurance: This method validation study was conducted in accordance with the spirit of US-FDA Good Laboratory Practices (GLP) regulations and OECD GLP guidelines but was not inspected for compliance. Animal Health Drug Metabolism Standard Operating Procedure (SOP) on validation of assay methods (Krabill, 1999) were used in this study.

Matrices: Liver and perirenal fat were collected from five non-treated control heifers. No animals had been treated with hormone implants or were pregnant. From three animals that had been treated with MGA, liver and fat samples were collected. These samples were selected since residue analyses using an experimental HPLC-UV assay indicated that they were expected to have MGA concentrations within the quantitation range of the developed method.

Analytical method description: In the fat assay, a 5-g tissue sample was dissolved/extracted with 10% ethylacetate in hexane (v/v) with heating, then partitioned into heated acetonitrile. Trideuterated MGA (d_3MGA) was added as internal standard. The acetonitrile phases were evaporated and the resulting residue reconstituted in hexane, further purified by solid-phase extraction using a solid phase extraction cartridge, the compounds being eluted with 60:40 hexane: acetone (v/v). The eluent was evaporated and the sample was reconstituted in 500µl methanol + 500µl water.

The extraction for MGA in liver was similar, only differing in the amount of tissue required (6g) and the solvent for dissolution (100% hexane, no heating)

The MGA residue concentration was measured by reverse-phase gradient HPLC with mass spectrometric detection using positive-ion electrospray ionization (ESI) mass spectrometry with selected-ion monitoring of the molecular ions ($M+H^+$). HPLC was performed in a Nucleosil C18 column (2 x 125 mm, 5 µm, integral guard column, injection volume 230 µL, flow rate 500 µL /min) with a solvent gradient (0.1% formic acid in water-acetonitrile, run time 15 min). For the MS detection, the instrument adquisition and integration parameters were: Q1 scan, unit resolution (0.7- 0.8 amu); scan width: m/z 200-450, 1 second adquisition; MGA integration window: m/z 396.9-397.6, i.e., $M+H^+$ of 397.24±0.35; d_3MGA integration window: m/z 399.9- 400.6, i.e., $M+H^+$ of 400.26 ±0.35. Signal optimization was performed manually by injection of solvent blank and selected standards to verify signal stability and signal intensities/peak areas. The MGA and d_3MGA peaks overlap with a mean retention time of 5.2 min

Calculation and expression of results: Calibration curves are constructed by weighted linear regression using concentration (x) and peak area ratios for all standards (y, ratio of MGA to d_3MGA). Residue concentrations were determined by interpolation.

Acceptability of standards curves: Twelve calibration standards were analyzed with solution concentrations of 2.55 ng/mL to 51.0 ng/mL in a random order at the beginning and end of each set of samples. Acceptability of the calibration curve is established by comparison of back-calculated concentrations for standards (interpolation from the calibration curve). Individual standards which deviate by more than 20% from expected values are excluded, the curve is

recalculated, and new back calculated concentrations are established until all remaining standards are 80-120% of expected values. If more than 25% of the standards must be excluded, the entire set (curve and samples) is rejected. Mean deviations for standards in the nine set of standards in this study ranged from 5.5% to 9.4% when standards with deviations > 20% are excluded. Only one set of samples (fat) was excluded and repeated due to and unacceptable standard curve.

Accuracy and precision

Recovery: For liver, tissue from control animals was fortified at 1.02, 2.04, and 3.06 µg/kg. For fat, samples were fortified at concentrations of 2.55, 5.10, and 7.65 µg/kg. These concentrations corresponded to approximately 50%, 100%, and 150% of the provisional MRLs for these tissues. Results are presented in the following table:

Fortification level in liver (µg/kg)	**LIVER : Mean percent recovery by day (n=3/day)**					**% Overall**		
	Set 1	Set 2	Set 3	Set 4	Set 5	Mean	Std.er.	CV
1.02	136	94.7	96.7	118	95.0	108	5.1	22.0
2.04	95.0	97.1	96.2	95.8	97.9	96.4	5.1	9.8
3.06	107	98.6	96.7	91.5	105	99.4	5.2	8.87
All levels	--	--	--	--	--	101	3.0	16.0
Fortification level in fat (µg/kg)	**FAT: Mean percent recovery by day (n=3/day)**					**% Overall**		
	Set 1	Set 2	Set 3	Set 4	Set 5	Mean	Std.er.	CV
2.55	91.8	88.9	90.0	118	88.9	95.5	4.7	16.8
5.10	95.4	99.8	84.7	98.7	97.3	95.2	4.7	12.0
7.65	89.9	104	74.7	97.6	101	93.3	4.7	14.3
All levels	--	--	--	--	--	94.7	3.5	14.2

In liver, there were no differences in MGA recoveries between the three fortification levels, though variance for the lowest fortification level (1.02 µg/kg) was greater than the other two levels (lower level approximates the LOQ of the assay). In fat, there were no differences in MGA recoveries and variance was homogeneous among the three fortification levels.

Repeatability and reproducibility. Using data for all fortification levels, precision within day and between day was calculated.

	Liver		**Fat**	
	%CV	95% Prediction limits (%)	%CV	95% Prediction limits (%)
Repeatability	13.7	27.8	12.0	22.8
Reproducibility	15.8	32.2	14.8	28.1

Given the variability of measurements of individual calibration standards (see acceptability of standard curves), the variability associated with MS analysis appears to be the principal factor affecting the accuracy and precision.

The analytical performance for samples with incurred tissues was similar to performance for fortified samples. For liver, CVs for 3 samples (duplicate, five days) were 6.18%, 5.67% and 8.99% (3.96, 3.08 and 7.29 µg/kg). For fat, CVs for 2 samples (duplicate, five days) were 21.0% and 17.3% (4.20 and 7.78 µg/kg). The third fat sample had residues < 0.5 µg/kg that were near the LOQ (0.42 µg/kg).

Specificity. Liver and fat tissues of five control animals were analyzed (n=30 for liver, n=27 for fat). No significant interference occurred in the MGA elution region of the chromatogram (5.0-5.5 min). Signal was predominantly baseline noise. In a small number of cases, a minor peak was detected attributed to MGA (d_3MGA also detected) and due to imminent failure of guard column (chromatographic artefacts).There was no positive bias at lower concentrations.

Quantitation range. LOD and LOQ in both tissues were estimated based upon the repeated analysis of blank tissues. LOD is defined as the mean + 3 SD. LOQ is defined as the mean + 10 SD.

For liver (n=30, mean = 0.16µg/kg, SD= 0.074µg/kg), estimates for LOD and LOQ were 0.38 µg/kg and 0.89 µg/kg, respectively. The lowest fortification level tested in the method validation was 1.02 µg/kg (approaches the LOQ)

For fat (n=27, mean = 0.17µg/kg, SD= 0.083µg/kg), estimates for LOD and LOQ were 0.42 µg/kg and 1.0 µg/kg, respectively. The lowest fortification level tested in the method validation was 2.55 µg/kg.

The upper limit of quantitation was arbitrarily defined as 51 ng/mL in solution (the highest concentration analyzed) and corresponds to 10 µg/kg in fat and 8.5 µg/kg in liver tissue equivalents.

Ruggedness

Analytical method: The ruggedness of sample preparation method was empirically evaluated by deliberately not controlling several experimental, equipment and procedural parameters. Magnitudes of repeatability and reproducibility were acceptable with this approach. It is unnecessary to control stringently the majority of experimental parameters, though the sponsors have listed recommendations for certain steps that should be controlled during the assay.

Stability of MGA and samples: MGA has proved to be stable in a variety of solvents and under a wide range of conditions. Sponsors mentioned that stability of homogenized/ground samples has been addressed previously (Merrit et al., 2000) indicating that there was no significant decline in recovery of MGA from samples subjected to two freeze/thaw cycles and no discernible change in MGA concentrations for samples stored for approximately 2 years at -10°C. Prepared samples stored in their original HPLC vials were proved to be stable up to four days at ambient laboratory conditions (lack of positive or negative bias, mean bias < 5%).

Applicability: The method provided had a simplified preparation for both tissues. It requires an HPLC-MS equipment and trideuterated melengestrol acetate as internal standard. No other special drug or expensive laboratory equipment is needed.

In addition to the sponsor's methods, a number of other methods with good performance characteristics have been developed. A variety of potentially suitable residue methods is available according to the requirements.

A method using GC-MS has been reported for the analysis of beef fat with an adequate LOQ (Neidert et al., 1990). Crude extracts were purified by thin layer chromatographic techniques. GC-MS analysis conditions has been described in the publication. The monitored masses were as follows: positive ions m/z $397^+$and 337^+ and negative ions m/z 396^- and 337^-. The electron impact mass spectrum of MGA, although producing characteristics fragments, was not useful for the confirmation of low level samples (< 10 µg/kg). Positive and negative chemical ionization with methane and isobutane produced multiple ions that allowed to confirm samples containing 1 µg/kg.

More recently, a study to provide data for the residues of MGA in plasma and edible tissues after normal and overdose treatment described a LC-MS method for quantification of residues in liver, kidney and muscle and a GC-MS method for quantification in perirenal fat (Daxenberger et al., 1999). After extraction, samples were defeated and cleaned on octadecyl solid-phase extraction cartridges. Quantification was performed using deuterated melengestrol acetate as internal standard. In the LC-MS analysis, the monitored ions after electrospray ionization (ES) were m/z 397, 438 and 337 for MGA and m/z 400, 441 and 349 for MGA-d3. For quantification the ions used were m/z 397 (analyte) and m/z 400 (internal standard). In the GC-MS analysis, purified extracts were derivatized with HFBA previous injection. The monitored ions after electrospray ionization (ES) were m/z 489, 533 and 592 for MGA and m/z 492, 536 and 595 for d_3MGA. For quantification the ions used were m/z 489 (analyte) and m/z 492 (internal standard). Both methods were properly validated. LOD were 5 µg/kg in fat, 1 µg/kg in liver and kidney and 0.5 µg/kg in muscle. These values allow to determine melengestrol residues in fat and liver at the established MRLs.

For the control of anabolic agents, a GC-MS sensitive method for detection of a wide range of anabolic steroids in liver and muscle, including melengestrol acetate, was developed (Marchand et al., 2000). After extraction of the lyophilized meat, solid phase extraction on a polymeric stationary phase was performed. Liquid-liquid partitioning was used to separate the analytes into two main categories: phenol containing molecules and Δ4-3-one containing molecules, such as MGA. Solid-phase extraction was performed before applying a specific derivatisation for each compound sub-group. All androgens and progestagens (as MGA) were derivatized using MSTFA-TNIS-DTT (N-methyl-N-(trimethylsilyl) trifluoroacetamide; trimethyliodosilane; dithiothreitol). The combination of high-resolution GC chromatography with a quadrupole mass spectrometer permitted detection of melengestrol at 1.5 µg/kg. The monitored ions were m/z 570 (melengestrol) and m/z 573 ($[^2H_3]$ melengestrol).

LC in combination with ion-trap mass selective detection (MS^n) is a powerful alternative to confirm the presence of MGA in kidney fat (Schwillens et al., 2000; Stolker et al., 2002). After sample pre-treatment, the analytes were extracted from the matrix by supercritical CO_2 and trapped directly in-line on alumina placed in the extraction vessel. The samples were analyzed using LC-MS^n detection and single ions of the analytes and internal standard (deuterated molecule) are monitored for quantification. For confirmation, two transition ions, one MS^2 ion m/z 337 and one MS^3 ion m/z 279 were monitored. Applying the EU-criteria to the measured relative intensities of the transition ions and comparing these with the relative intensities obtained for the standard, the presence of MGA can be confirmed at a level of 0.5 µg/kg.

A sensitive enzyme immunoassay (EIA) for the determination of melengestrol acetate (MGA) in adipose and muscle tissues has also been developed (Hageleit et al., 2001). After extraction of the samples with petroleum ether and purified octadecyl-silica-cartridges, a competitive microtitration plate enzyme immunoassay is applied. The efficiency of this screening method was demonstrated by direct comparison to GC-MS and LC-MS methods. The LOD for fat was 0.4 µg/kg and for muscle 0.05 µg/kg, much lower than the fixed MRLs.

APPRAISAL

A HPLC-MS method was developed and va1idated to quantify residues of melengestrol acetate (MGA) in bovine fat and liver tissues. This method was developed to support temporary MRLs for MGA of 5 µg/kg in fat and 2 µg/kg in liver established by the 54th meeting of the Committee. MRLs were not established for other tissues.

The validation study was conducted in accordance of Good Laboratory Practices (GLP) but was not inspected for compliance.

In the fat assay, a 5-g sample was dissolved/extracted with 10% ethyl acetate/hexane with heating, partitioned into acetonitrile, and further purified by a solid-phase extraction cartridge. The MGA residue concentration was measured by gradient reverse-phase HPLC with mass spectrometric detection (positive-mode electrospray ionization). MGA ion is monitored (m/z 396.9-397.6). Trideuterated MGA (d_3MGA) was utilized as an interna1 standard. The assay for MGA in liver was similar, with the method only differing in the amount of tissue sample required (6 g) and the extraction process.

The following criteria were evaluated: acceptability of standard curves, quantitation range, accuracy, repeatability and reproducibility, assay specificity, and assay ruggedness..

Based upon the recovery of MGA from fortified samples, the accuracy of the assay was acceptable. Mean recoveries of MGA were of 94.7% (CV: 14.2%) for fat and 101% (CV:16%) for liver. The coefficients of variation for repeatability and reproducibility of analysis were <16% for both tissue types. The analytical performance for samples with incurred residues was similar to performance for fortified samples. No MGA false positive was observed in tissue samples from five different animals and there was no positive bias to data at lower concentrations.

The limit of detection (LOD) and limit of quantitation (LOQ) were estimated for both assays. LOD and LOQ for the liver assay were 0.38 µg/kg and 0.89 µg/kg, respectively. LOD and LOQ for the fat assay were 0.42 µg/kg and 1.0 µg/kg, respectively. Given these estimates for LOQ, the lowest fortification level used in this study for liver (1.02 µg/kg) approximated the computed LOQ, whereas the lowest fortification level for fat (2.55 µg/kg) was greater than the computed LOQ.

Ruggedness and applicability of the method are adequate. HPLC-MS equipment and trideuterated melengestrol acetate as internal standard are required. No other special drug or expensive laboratory equipment is needed.

The estimated limits of quantitation for MGA in fat and liver were 20% and 50% of the provisional MRLs established by JECFA for MGA in bovine fat and liver tissues. Others methods with good performance characteristics are also available.

The Committee considered that the analytical method submitted was validated for purpose. The Committee recommended permanent MRLs of 5 µg/kg for bovine fat and 2 µg/kg for bovine liver respectively.

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