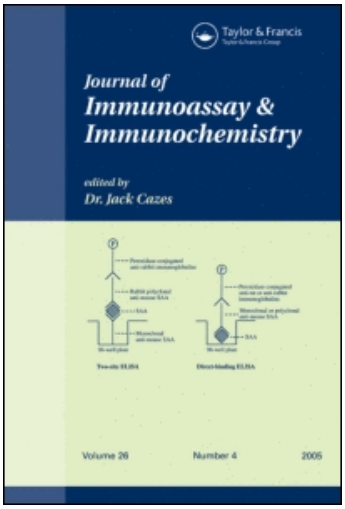

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ABSTRACT

For eight weeks, two heifers each had been orally administered daily doses of 0, 1.5, or 5 mg melengestrol acetate (MGA) in a feed premix. Four heifers received the labeled dose of 0.5 mg/day. Regular feces samples were taken throughout the experiment. A rapid screening method for the determination of MGA in feces was developed, consisting of liquid extraction, clean-up on solid-phase extraction cartridges and quantification by enzyme immunoassay (ELISA). Residues in feces were dose-dependent with mean values of < 0.25,

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2.0, 4.4, or 15.4 ng/g for 0, 0.5, 1.5, and 5 mg/day doses, respectively. In contrast to urine analysis, feces analysis appeared to be a suitable means of non-invasive screening before slaughter for surveillance of MGA treatment and verification of its compliance with labeled dosage.

Key Words: MGA; Melengestrol acetate; Anabolic; Feces; Screening; Residue analysis.

INTRODUCTION

The use of veterinary drugs in animal fattening can be monitored by residue analysis of edible tissues. Post-mortem analysis, however, does not prevent illegally treated animals from being slaughtered. Nowadays, in surveillance of food production, the use of methods is encouraged by which the manufacturing process, and not the final product, is controlled. For this purpose, residue analysis protocols have been developed for urine and feces — matrices that can easily be sampled *in vivo* by non-invasive procedures. In cattle fattening, for example, the use of anabolic steroid hormones like androgens,[1,2] glucocorticoids,[3,4] estrogens,[5,6] and gestagens[7] can be identified via fecal metabolites. Melengestrol acetate (MGA) is a synthetic gestagen, which is used for estrus synchronization[8] and growth promotion[9] of heifers. Its affinity to the bovine progestin receptor is more than 5-fold higher than that of the natural ligand progesterone.[10] Its mode of anabolic action is mediated by sustained estradiol production by the continually developing follicles, which are prevented from ovulation.[11] In the USA and some other beef-producing countries, MGA is licensed as growth promoting agent for the fattening of heifers, whereas, in the European Union, natural and xenobiotic sex hormones are generally banned as growth promoters.[12] In the USA, a tolerance level of 25 ng MGA/g fat[13] shall assure that contaminated carcasses do not enter the food supply. In Canada, an administrative action level of 30 ng/g fat[14] was allocated for this purpose. The recommended maximum residue limits (MRL) suggested by the Joint Expert Committee for Food Additives and Contaminants (JECFA) are 2 ng/g liver and 5 ng/g fat.[15] These lower residue limits leave a small margin towards the actual residues after correct application, as it was shown that, already, after labeled dose application, 5 ng/g in fat are exceeded.[16] The intention of this study was to develop a screening method for the surveillance of MGA administration in the living animal by means of feces analysis.

EXPERIMENTAL

Animal Experiments

All animal experiments have been conducted within the scope of previous studies on residue formation in edible tissues[16] and the mode of anabolic action of MGA after different dosages.[11] Briefly, two Holstein-Friesian heifers each received daily doses of 0 mg (control group), 0.5, 1.5, or 5 mg MGA (ICN Biochemicals, Eschwege, Germany) in a 500 g ration of bruised grain every morning 56 days prior to slaughter. Additionally, two animals were fed with the labeled dose of 0.5 mg/day for 56 days, but treatment was suspended two days before slaughter. Feces samples were collected twice a week, every 3.5 days, and analyzed during weeks 1–2, 4–5, and 7–8. The samples were taken alternately in the morning and in the evening, reflecting different periods of intestinal passage of the MGA feed premix.

Determination of MGA

MGA in feces was quantified by enzyme immunoassay (ELISA) after liquid extraction and solid-phase clean-up as described previously.[17] Fecal samples were prepared as follows: 1 g feces was suspended in 2 mL purified water and extracted with 8 mL petroleum ether (Baker 8115, Phillipsburg, NJ) in 20 mL glass tubes by shaking for ½ h at room temperature (horizontal shaker Edmund Bühler SM25, Hechingen, Germany). After centrifugation at 900 g and −15°C for 15 min (centrifuge Sigma 6 K 10, Osterode am Harz, Germany), the organic phase was decanted and evaporated at 60°C in a shaking waterbath (GFL 1083, Großburgwedel, Germany). Residues were redissolved in 1 mL methanol. For clean-up, the extract was diluted with 2.5 mL purified water and loaded onto an octadecyl-silica-cartridge (Bakerbond SPE∗ Octadecyl (C18)-column, Baker 7020-01, Phillipsburg, NJ, USA), which had been equilibrated with 20 mM Tris-HCl-buffer pH 8.5/methanol 80/20 v/v (equilibration buffer). After washing the cartridge with 2 × 1 mL equilibration buffer, and 2 × 1 mL methanol/water 40/60 v/v, MGA was eluted with 1 mL methanol/water 80/20 v/v. The eluate was diluted with 1 mL purified water. For quantification of MGA concentrations by ELISA, a further dilution with methanol/water 40/60 v/v by 1:5 was necessary. The ELISA procedure was performed following a standard protocol as previously described.[17] The detection limit was

calculated as the mean signal of 22 negative control samples plus three standard deviations.

Method Validation

For a detailed validation of the extraction and clean-up protocol, pooled negative feces samples from untreated heifers were fortified with different amounts of MGA reflecting the concentrations occurring after labeled and over-dosed treatment with MGA. In feces, mean recoveries of 0.3, 0.6, 2.4, and 9.6 ng/g were 134%, 116%, 89%, and 90%, respectively, with a mean inter-assay variation of 16%. The detection limit was 0.25 ng/g feces. In addition, the ELISA results were confirmed by comparison with the results of liquid chromatography-mass spectrometry (LC-MS) as described previously.[18] One morning and one evening feces sample each from all eight treated animals was measured both by LC-MS and by ELISA. The concentrations measured by the ELISA screening method were 79% of those measured by the LC-MS confirmation method, with a high linear correlation (coefficient of correlation $r = 0.99$).

RESULTS

Concentrations of MGA in feces were dose-dependent. Mean residues after 3-fold and 10-fold dose were 2.3-fold and 7.9-fold higher, respectively, than after regular 1-fold dose treatment (Table 1). It appeared that, in morning samples (i.e., 24 hours after the last MGA application), MGA concentrations were higher than in evening sampling,

Table 1. MGA residues in feces given as mean of all samples ± standard deviation.

	Dosage (mg MGA per day)			
Sampling time	0	0.5	1.5	10
All samples ($n = 78$)	< 0.25 ng/g	2.0 ± 0.6 ng/g	4.4 ± 1.2 ng/g	15.4 ± 3.7 ng/g
Morning samples ($n = 44$)	< 0.25 ng/g	2.0 ± 10.6 ng/g	4.8 ± 1.2 ng/g	16.4 ± 3.6 ng/g
Evening samples ($n = 24$)	< 0.25 ng/g	1.9 ± 0.6 ng/g	3.9 ± 0.9 ng/g	14.2 ± 3.8 ng/g

reflecting the period necessary for intestinal transport after oral ingestion. In all samples from treated animals, MGA was detectable, whereas no MGA was detected in samples from untreated animals. The mean concentration after application of labeled dose (0.5 mg/day) was 7-fold higher than the limit of detection.

DISCUSSION

A rapid screening method for the determination of MGA-treatment of heifers in feces was developed using a validated ELISA for the quantification after liquid extraction and reversed phase (RP-18) solid-phase clean-up. The results of ELISA screening of feces showed very good correlation with the results obtained by LC-MS.[18] The method described for feces is suitable for the screening of correct application of the labeled dose of 0.5 mg of MGA per day and head. Assuming a threshold level for MGA in feces of 2.6 ng/g (= mean of 2.0 ng/g plus standard deviation of 0.6 ng/g) for correct dosage, there would have been 10% false non-compliant results. The 92% of those animals treated with 1.5 mg MGA, and all samples of animals fed with 5 mg MGA per day, would have been correctly identified as non-compliant. In urine, of which samples had been taken twice a week as well, concentrations of MGA or cross-reacting MGA metabolites were below the detection limit of 0.02–0.06 ng/mL in all samples throughout the experiment (data not shown) and did not differ from control sample values. Obviously, the concentrations of parent compound plus cross-reacting metabolites were below the ELISA detection limit yet even after deconjugation by glucuronidase/sulfatase. Three main metabolites of MGA produced by liver microsomes in cell culture[19] have cross-reactivities to the applied antiserum between 55% and 100%.[17] Our results confirm previous radioactive tracer studies, which showed bile to be a primary excretion route of MGA, as only 9% of the radioactivity was found in urine.[20] Furthermore, it was reported that about 10–17% of the applied dose of MGA passed the intestinal tract unabsorbed.[20] Therefore, urine is not a suitable matrix for the surveillance of MGA administration. The concentrations of MGA in feces reflect the applied dose in a similar manner as concentrations in plasma and in edible tissues.[16] Plasma levels after 3-fold dose were 3.2-fold higher than after 1-fold dose, and 7-fold higher after 10-fold dose; in fat factors were 3.9 and 7.9, respectively. Therefore, it should be possible to extrapolate potential infringement of maximum residue limits or tolerances of residues in edible tissues from fecal concentrations. According to a study performed

by Daxenberger et al.[16] with GC-MS (fat) and LC-MS (liver) after internal standardization with MGA-d3, the MRL of 2 ng/g liver[15] may be exceeded after 3-fold dose treatment with 1.5 mg MGA/day (mean concentration of MGA = 5.0 ng/g) while, in fat (mean MGA = 7.5 ng/g), even the use of the regular dose of 0.5 mg/day may result in infringement of the MRL of 5 ng/g.[15] On the assumption that these results are confirmed by future studies, fecal MGA concentrations exceeding 2.6 ng/g might indicate infringement of MRL in edible tissues. Finally, it should be mentioned that the potential infringement of the suggested MRL after application of 0.5 mg MGA per head and day should be regarded by international boards (Codex Alimentarius). Application of lower doses would circumvent this problem but, if so, the effectiveness of MGA for anabolic purposes is questionable. In conclusion, the use of MGA in compliance with maximum labeled dosage can be monitored before slaughter by determination of residues in feces. The ELISA used for this purpose herein was validated for edible tissues and plasma,[17] and is commercially available (r-biopharm, Darmstadt, Germany). The results of the screening method were confirmed by LC-MS. Exceeded threshold values indicate an overdosed application and can be used to prevent the suspect animals from being slaughtered and marketed.

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