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Confirmatory analysis of acetylgestagens in plasma using liquid chromatography–tandem mass spectrometry

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Abstract

A confirmatory method has been developed and validated for the determination of chlormadinone acetate (CMA), megestrol acetate (MGA), melengestrol acetate (MLA) and medroxyprogesterone acetate (MPA) in bovine and porcine plasma. Analytes are extracted from plasma samples using matrix-assisted liquid–liquid extraction (LLE) on Extrelut NT columns followed by C18 solid-phase extraction (SPE). Analytes were analysed using liquid chromatography–tandem mass spectrometry (LC–MS/MS), and quantification was performed using matrix-matched calibration standards in combination with deuterated internal standards. In accordance with Commission Decision 2002/657/EC, two ion transitions were monitored for each analyte. Decision limits (CCα) were estimated by analysing 20 blank plasma samples and ranged from 0.1 to 0.2 ng mL^{-1}. Detection capabilities (CCβ) were estimated using 20 plasma samples fortified at 0.5 ng mL^{-1} and were <0.5 ng mL^{-1}. In the range 0.5–2 ng mL^{-1}, the mean intra-laboratory reproducibility of the analytes ranged from 6 to 18% (%R.S.D.). Analytes were shown to be stable in fortified plasma samples for >8 months when stored at −20 °C.

Keywords: Gestagens; Liquid chromatography–tandem mass spectrometry (LC–MS/MS); Monitoring; Plasma; Residues; Steroids

1. Introduction

Acetylgestagens, such as medroxyprogesterone acetate (MPA), melengestrol acetate (MLA), megestrol acetate (MGA) and chlormadinone acetate (CMA), are synthetic progestagens (Fig. 1) that can be used as growth promoters for the fattening of cattle and swine. However, as the consumption of animal tissues containing hormone residues may pose a risk to consumer safety [1], the use of acetylgestagens as growth promoting agents in livestock production is prohibited in the European Union [2]. Illicit administration of acetylgestagens to cattle has previously been reported in several European countries [3,4]. Therefore, analysis of acetylgestagens is included in the Danish monitoring programme for veterinary drug residues.

Similarly to other anabolic steroids, gestagens are lipophilic and therefore accumulate in adipose tissues [5]. Consequently, kidney fat is the matrix of choice in many of the analytical methods that have been described for monitoring the illegal use of gestagens [6–11]. In these studies, gestagens were extracted from fat using techniques such as liquid–liquid extraction (LLE), solid-phase extraction (SPE), supercritical fluid extraction (SFE), accelerated solvent extraction (ASE) and matrix solid phase dispersion (MSPD). Extracts were then analysed using enzyme immunoassay (EIA), gas chromatography–mass spectrometry (GC–MS) or liquid chromatography–mass spectrometry (LC–MS). These analytical techniques have also been used for the analysis of gestagens in matrices such as muscle, liver, kidney, faeces, urine, and plasma [3,7,12].

Although regulatory control of the illegal use of hormonal growth promoters is possible through residue analysis of edible tissues such as muscle, liver, and kidney fat, these matrices do not allow for monitoring at the farm level. However, matrices such as plasma, hair, urine and faeces can be sampled *in vivo*, enabling the detection of illegally treated animals prior to slaughter. As growth-promoting treatment with illegal substances might intentionally be stopped to allow for pre-slaughter drug withdrawal, national monitoring programmes should also include *in vivo* sampling as a means to uncover illicit drug administration.

Steroid abuse is commonly detected in urine samples. However, earlier studies have shown that urine is not a suitable matrix for monitoring the administration of acetylgestagens, as very little of the parent compound or its metabolites are

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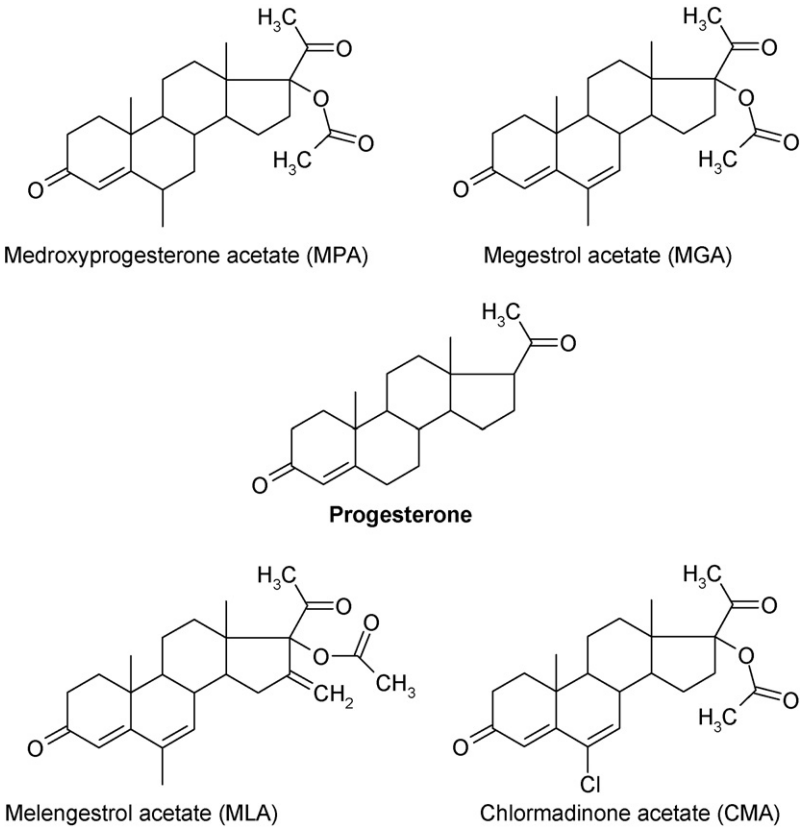

Fig. 1. Chemical structures of progesterone and relevant synthetic analogues.

excreted via the urine [3,5,12]. On the other hand, faeces and hair analysis have been shown to be appropriate for the non-invasive monitoring of acetylgestagens [12,13]. Another matrix that can be used for monitoring at the farm level is plasma. Several analytical methods for the determination of gestagens in plasma have previously been published [7,14–16]. In contrast to the methods described for hair and faeces analysis, sample clean up for plasma is simpler and less time-consuming. In the past, acetylgestagens were detected in plasma using GC–MS [14] and EIA [7]. Although both techniques provide low limits of detection, GC–MS involves time-consuming derivatization steps and EIA does not provide confirmatory identification. Therefore, recent methods for the determination of acetylgestagens in plasma involve LC–MS techniques.

Previously published liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods have described the analysis of MPA in human and porcine plasma [15,16]. In contrast to these methods, the confirmatory method developed in this study enables the simultaneous determination of four acetylgestagens in bovine and porcine plasma. Finally, the method was validated in accordance with Commission Decision 2002/657/EC [17].

2. Experimental

2.1. Reagents and chemicals

Standards of MPA, MGA and CMA were purchased from Sigma–Aldrich (St. Louis, MO, USA), MLA was bought from Steraloids Inc. (Newport, USA) and the deuterated internal standards (MPA-d_3, MGA-d_3 and MLA-d_3) were from RIVM (Bilthoven, The Netherlands). Stock solutions of every compound were prepared separately in ethanol at concentrations of 1 mg mL^{-1}. Stock solution of internal standards, however, were prepared by reconstitution of ampoules containing a fixed amount of material with ethanol at a concentration of 0.1 mg mL^{-1}. Stock solutions were stored at −18 °C until use for up to 1 year. A final working solution containing all the compounds at 0.1 μg mL^{-1}, except internal standards, was prepared by diluting stock solutions with ethanol. A final working solution of internal standards containing all three internal standards at 0.1 μg mL^{-1} was also prepared by diluting stock solutions with ethanol. Final working solutions were freshly made.

All reagents were of analytical or HPLC grade and supplied by Merck (Darmstadt, Germany) and Rathburn Chemicals (Walkerburn, Scotland). Water was ultra-purified using a Maxima purification system from USF Elga (Bucks, UK).

2.2. Samples

As part of the national plan for monitoring drug residues in animals and animal products, blood samples were collected at slaughterhouses, and samples from live bovines were taken from livestock at the farm level. Sample containers, containing an anticoagulating reagent (heparin), were sent to sample collectors. As soon as the samples were received, they were prepared and the plasma was stored at −18 °C until analysis.