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Ultra high performance liquid chromatography/tandem mass spectrometry based identification of steroid esters in serum and plasma: An efficient strategy to detect natural steroids abuse in breeding and racing animals

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ABSTRACT

During last decades, the use of natural steroids in racing and food producing animals for doping purposes has been flourishing. The endogenous or exogenous origin of these naturally occurring steroids has since remained a challenge for the different anti-doping laboratories. The administration of these substances to animals is usually made through an intra-muscular pathway with the steroid under its ester form for a higher bioavailability and a longer lasting effect. Detecting these steroid esters would provide an unequivocal proof of an exogenous administration of the considered naturally occurring steroids. A quick analytical method able to detect at trace level (below 50 pg/mL) a large panel of more than 20 steroid esters in serum and plasma potentially used for doping purposes in bovine and equine has been developed. Following a pre-treatment step, the sample is submitted to a solid phase extraction (SPE) before analysis with UPLC–MS/MS. The analytical method's efficiency has been probed through three different *in vivo* experiments involving testosterone propionate intra-muscular administration to three heifers, 17-estradiol benzoate intra-muscular administration to a bull and a heifer and nandrolone laurate intra-muscular administration to a stallion. The results enabled detecting the injected testosterone propionate and 17-estradiol benzoate 2 and 17 days, respectively, post-administration in bovine and nandrolone laurate up to 14 days post-administration in equine. The corresponding elimination profiles in bovine serum and equine plasma have been established. The first bovine experiment exhibited a maximal testosterone propionate concentration of 400 pg/mL in one of the three heifer serum within 5 h post-administration. The second bovine experiment reported a maximal 17-estradiol benzoate concentration of 480 pg/mL in the same matrix recorded 9 days after its administration. The last equine experiment resulted in a maximal nandrolone laurate concentration of 440 pg/mL in horse plasma 24 h after administration.

1. Introduction

The use of androgenic anabolic steroids (AAS) as growth promoters in food producing animals is prohibited in the European Union according to the EU Directive [1]. Their use is also forbidden for equine performance enhancement according to article 6 of IFHA [2]. The latter bans the use of substances capable of giving horses an advantage, contrary to the horse's inherent merit. Moreover, the use of these substances is supposed to result in biological [3,4] and behavioral [5,6] modifications and can be considered dangerous for the animal [7,8].

A consequent number of methods permitting the detection of those substances and their subsequent metabolites, based essentially on liquid or gas chromatography along with mass spectrometry, have been developed and implemented at the different doping control laboratories around the world [9,10]. These methods succeed in covering a wide range of xenobiotic substances and their metabolites through their detection at trace levels thanks to improved instrumentation sensitivity [11,12].

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Although very low detection levels have been reached recently, the anti-doping world is still facing some issues. One of the most challenging concerns lies in the determination of endogenous AAS fraudulent use. With regard to their natural existence in the organism, it is difficult to distinguish between the detected signal corresponding to the administered steroid and the one arising from endogenous steroid production of the animal. The challenge is even greater taking into account the wide diversity in the rates of natural steroid biosynthesis for different individuals of the same species due to environmental and biological factors [13]. Consequently, thresholds in concentrations and ratios of endogenous steroids were adopted [14,15]. Although being efficient in most cases, these thresholds face some limits. In the case of nandrolone doping detection in equine, the established urinary concentration ratio threshold of 5α-estrane-3β,17α-diol/5(10)-estrene-3β,17α-diol above 1 [16,17] is often overpassed for a consequent number of urinary samples taken from intact male horses during routine analysis leading to high number of false suspicious results.

These observations made necessary the development of approaches alternative to direct targeted detection of active doping agents and their metabolites. Many approaches have therefore recently been investigated such as metabolomics [18,19] for screening purposes, or IRMS technique [20,21] for confirmatory purposes which, despite their promising results, are time and money consuming for anti-doping laboratories.

Testosterone, nandrolone and boldenone are considered to be the main endogenous AAS used for doping purposes in equine and bovine [22–24]. These substances are usually administered as their synthetically produced esters by intramuscular injection [25] in order to exhibit a longer lasting effect [26]. Once the steroid ester is administered, it undergoes a progressive enzymatic hydrolysis in blood [27] to produce sustained doses of the active substance. Meanwhile, the blood stream enables the transmission of newly hydrolyzed steroids to their corresponding hormonal receptors. The period lasting effect of steroid esters depends on their side chain length; the longer the side chain, the slower its hydrolysis will be and vice versa. This difference in hydrolysis time between short and long side chain esters is a direct consequence of the interactions occurring between the steroid ester and its lipophilic excipient. Long ester chains are indeed more lipophilic than shorter chains and hence present a stronger interaction with their respective excipient. Consequently, their deliverance from the excipient to the plasma or serum is slower. This explains the commercialization of steroid ester cocktails formed of long and short side chain esters mixtures allowing reaching constant doses of active molecules on a wide time scale [28].

In this context, detecting synthetic steroid ester in blood gives an absolute proof of an exogenous administration of the suspected natural occurring steroid. Although these esters are quickly hydrolyzed by blood esterase, trace levels are remaining in the blood circulation for a relatively short time.

A number of previous works reported the steroid esters analysis in biological matrices and most of them dealt with hair as a reference matrix [29] because of its higher storage capability in steroid esters. Apart from being a relatively clean biological matrix and hence more adequate for analytes extraction, blood matrix exhibits higher amount of information than hair in free steroids and subsequent metabolites. As a consequence, several studies have been done for both free steroid and steroid esters extraction from blood [30–33]. Although these protocols succeeded in detecting specific steroid esters, some improvements were still needed for better monitoring of these substances especially in terms of sensitivity due to the trace levels present in blood. Moreover, some of these methods were time consuming [34] or focusing on one specific or a small set of steroid esters [35] which render their application limited to a certain extent.

The aim of the present study was to develop a quick, multi-residue and highly sensitive analytical method permitting the detection of 20 steroid esters at trace levels in equine plasma and bovine serum based on a UPLC–MS/MS instrumentation system. The developed method has been probed through three in vivo animal experiments consisting in an intra-muscular administration of testosterone propionate, 17-estradiol benzoate and nandrolone laurate, respectively, to three heifers, to a bull and a heifer and to a stallion.

2. Experimental

2.1. Animal experiment

The first animal experiment was conducted in Oniris (France) breeding facilities and in agreement with animal welfare rules and approval of Oniris ethics committee. Three heifers (bread Holstein) A, B and C were treated with an intra-muscular injection (1 mL) of Testos-P 100, a commercial oily testosterone propionate solution (100 mg/mL) of PharmaCom Ltd® (Batch 6544234). Serum samples were collected during the 5 days preceding the administration and 5 h, 1, 2, 6, 8, 10, 15, 20 and 27 days after administration. The samples were kept frozen in test tubes at −20 °C until analysis.

The second animal experiment was conducted in Afsca Food Safety Center (Belgium) breeding facilities in agreement with animal welfare rules and approval of Afsca ethics committee. A bull and a heifer of 18 months weighing 400 kg were treated with an intra-muscular injection (1 mL) of an oily homemade solution of 17-estradiol benzoate (80 mg/mL). Serum samples were collected during 7 days preceding the administration and 1, 3, 5, 7, 9, 13, 17, 21 and 24 days after administration. The samples were kept frozen in test tubes at −80 °C until analysis.

The third animal experiment was conducted in Coye-la-Forêt (France) in agreement with animal welfare rules and approval of France Galop (France) ethics committee. One stallion of 11 years old weighing 570 kg was treated with an intra-muscular injection (30 mL) of Laurabolin®, a commercial oily nandrolone laureate solution (20 mg/mL) of inervet® (Batch A106A07). Plasma samples were collected over the 6 days preceding the administration and all along the experiment until day 96 after administration. The samples were immediately centrifuged after collect to obtain fresh plasma. 5 plasma aliquots of 5 mL of each collected sample were kept frozen in test tubes at −80 °C until analysis. Urine samples were automatically collected along with plasma samples.

2.2. Chemicals and reagents

The different steroid esters reference standards used in the present study (testosterone acetate (TAc), testosterone propionate (TPr), testosterone benzoate (TBz), testosterone cypionate (TCy), testosterone enanthate (TEn), testosterone decanoate (TD), nandrolone propionate (NPr), nandrolone phenylpropionate (NPhPr), nandrolone benzoate (NBz), nandrolone decanoate (ND), nandrolone laureate (NL), boldenone propionate (BPr), boldenone benzoate (BBz), boldenone undecylenate (BUn), estradiol propionate (EPr), estradiol 17-benzoate (E17Bz), estradiol enanthate (EEn), estradiol cypionate (ECy), estradiol valerate (EV) and estradiol decanoate (ED)) were obtained from either Steraloids Inc. Ltd. (London, England) or Sigma–Aldrich (St. Quentin Fallavier, France). Norethindrone acetate (North), testosterone benzoate-d3 (TBz-d3) and testosterone decanoate-d3 (TD-d3) were used as internal standards (IS) and were obtained kindly as a gift from RIKILT (Wageningen, The Netherlands). Norgestrel (Norg), used as an external standard (ES), was purchased at Sigma–Aldrich (St. Quentin