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Disturbance in sex-steroid serum profiles of cattle in response to exogenous estradiol: A screening approach to detect forbidden treatments

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ABSTRACT

Estradiol benzoate (EB) has been one of the most widely used estrogenic agents in animal husbandry, as a way of exogenously introducing the natural hormone estradiol-17β into the animal organism. Estradiol was previously employed to induce anabolic effects or reproductive improvements in cattle. However, the employment of EB in European countries has been permanently forbidden by Directive 2008/97/EC to guarantee consumers' health. Despite this prohibition, the control of estradiol-17β and its esters continues to be a difficult task for residue-monitoring plans in European Communities because official analyses of natural thresholds for hormones in cattle have not yet been established, leading to a lack of confirmation for any exogenous administration of natural hormones. Several researchers have worked on excretion profiles of metabolites, variation in specific hormonal ratios and metabolomic fingerprints after hormonal treatments. This research focuses on the possible existence of disturbances in the serum profile of animals treated with EB in terms of steroid sex hormones (androgens, oestrogens and progestogens), by investigating the serum levels of several of these hormones. The serum samples were collected from three groups of cows: one treated with an intramuscular injection of EB, one treated with a combination of intravaginal EB and progesterone and a control (non-treated) group. The samples have been analysed by a validated high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method, and 17 natural hormones were identified and quantified. Subsequently, data from the serum profiles were submitted for statistic and multivariate analysis, and it was possible to observe a manifest variation between animal groups. The obtained results can help in the development of a viable screening tool for monitoring purposes in cattle.

Throughout history, humans have bred animals to accommodate increasing human requirements in terms of food and other products, such as leather and wool, the demand for which is particularly important in developed countries. A large number of substances, both natural and synthetic, have been applied in stock farming to speed up and improve animal growth, and to decrease feed costs [1,2]. Among these veterinary drugs, there are some natural hormones and substances with hormonal effects that have been applied to animals for different purposes, especially as growth promoters and as fertility regulators in cattle. The steroid hormones, which are steroids acting as hormones, contain the sex hormones oestrogens, gestagens and androgens (EGAs), and the corticosteroids. Although these hormones have a wide variety of applications within the veterinary field, they have been used in animal fattening due to the anabolic effect that increases weight gain in treated animals, and induces changes that are generally characterised by lower fat content and more lean mass [3–5]. The zootechnical use of some sex hormones, such as estradiol or its esters (i.e., estradiol benzoate (EB)), which successfully regulate oestrus in cattle, has also led to important improvements and financial gain in stock farming [6].

There have been several European regulations regarding the use of EGAs as animal growth promoters because of their possible toxic effect on public health. In the Council Directive 96/22/EC [7], the European Union prohibited the administration of substances having thyreostatic, oestrogenic, androgenic or gestagenic effects and of beta agonists in animal husbandry, while certain therapeutic applications of these drugs were still allowed. These anabolic steroids are included in group A substances according to Annex I of Directive 96/23/EC [8], which pertains to growth-promoting agents abused in animal fattening and unauthorised substances with no maximum residue limit (MRL). A zero-tolerance policy has been adopted, and especial analytical requirements have been stated in regard to these hormones [9]. In particular, estradiol-

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17β, used with the aim of promoting animal growth, was deemed as a complete carcinogen by the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) [10]. Estradiol-17β exerts both tumour-initiating and tumour-promoting effects, and the data currently available do not make it possible to obtain a quantitative estimate of the risk. Although Directive 2003/74/EC, amending Directive 96/22/EC, permanently prohibited the use of estradiol-17β and its ester-like derivatives as growth promoters, a temporary exemption was given until 14 October 2006 for their use as an oestrous-induction tool in cows, horses, sheep or goats. As alternative effective products exist and are implemented in the market [11], the European Parliament banned estradiol-17β and its ester-like derivatives, including those with a therapeutic purpose in farm animals, in 2008, to ensure human health protection within the European Community [12]. However, the possibility of widespread abuse of hormonal substances by unscrupulous farmers and veterinary professionals in some parts of Europe still exists, mainly due to the economic benefits these substances provide in animal husbandry [13].

The control of growth promoters in meat-producing animals is probably one of the most challenging tasks in the field of European residue-monitoring plans, as it involves a wide number of target substances, the variability of their chemical structures and their concentration levels and biological matrices used in residue surveillance [1,4]. With regard to the confirmation of use of xenobiotic analogues of natural sex steroids and non-steroidal compounds such as stilbenes and zeranol, there is an extensive range of successful methods that has been performed on different analytic matrices that have made the confirmation of illicit administrations of anabolics in cattle feasible [2,14–17]. However, hormones of natural origin, such as estradiol-17β, testosterone (T) or progesterone, are still a weak area in residue-monitoring plans due to their endogenous origin, as the target compound is always present. In such a case, the confirmation of an exogenous administration involves logical difficulties associated with distinguishing an exogenous origin from an endogenous (naturally occurring) presence of these hormones. In fact, it has been found that treatments with T or estradiol in bovines lead to equal or lower plasma concentrations of these compounds [18,19]. On the other hand, exogenous natural hormones are usually administered as simple semi-synthetic esters (i.e., 17β-estradiol benzoate and T decanoate), and a subsequent rapid hydrolysis of these compounds takes place as soon as they reach the bloodstream, where they generate non-esterified forms that are indistinguishable from naturally occurring forms [20]. These exogenous compounds follow the same pathways as the natural compounds biosynthesised by the animal, making the detection and confirmation of their exogenous administration difficult, if not impossible. These circumstances have led to the lack of success in detecting EB in serum or plasma, which has only been confirmed in hair from animals treated with this ester [14,21]. In addition, the demonstration of an exogenous administration of natural steroids, for instance, T, estradiol or cortisol, remains problematic, as no official threshold has been stated for natural hormone concentrations, mainly due to the fact that concentrations of naturally occurring hormones depend on the type of animal product, breed, gender, age, disease, medication and physiological condition [22]. Furthermore, no list of discriminative marker metabolites has ever been stated, accepted or published by the community reference laboratories (CRLs) or by the European Commission, regarding the misuse of natural hormones in stock farming.

The development of methods to provide unequivocal discrimination between the natural presence of an endogenous hormone and its presence as a consequence of an illegal exogenous administration remains a challenge. Some promising analytical approaches have been published in the past few years regarding this critical point of controlling residues in food of animal origin. The measurement of $^{13}C/^{12}C$ ratios by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) can be a powerful tool to trace the true origin of steroids, and is one of the most promising approaches for the control of exogenous administration of natural hormones, as it has already been applied for anti-doping in sports [23,24]. In recent years, the potential of '-omic' technologies (metabolomics, proteomics with transcriptomics) coupled with bioinformatics has been investigated for the development of reliable molecular biomarkers, and to obtain discrimination based on targeted profiling of metabolites [25–27]. Other research has focussed on the variation of the excretion profile of phase II metabolites as a consequence of exogenously administered steroids [28,29], and in variation of some urinary or plasmatic metabolites from the biosynthetic pathway of sex hormones [18,30], or, even in blood chemistry [31].

Summing up, more information concerning steroid levels in animals treated with natural hormones seems necessary to establish acceptable thresholds of natural hormones or for use as a screening approach in residue-monitoring plans. In the present study, an analytical evaluation of serum profiles of several natural hormones from the biosynthetic pathway of sex hormones has been performed to prove the existence of any disturbance in the serum profile in response to exogenous estradiol administration in cattle. Bovine serum samples were analysed using a method based on liquid chromatography–tandem mass spectrometry (high-performance liquid chromatography tandem mass spectrometry, HPLC–MS/MS) [32], previously validated according to Decision 657/2002/EC criteria [9]. The samples were obtained from cows treated intramuscularly with the main ester of natural estradiol, 17β-estradiol benzoate, and animals treated with a common intravaginal combination of 17β-estradiol benzoate and progesterone. Serum from non-treated animals, which were used as control animals, was also collected. Free plasma concentrations of 17 natural steroids belonging to the three existing groups of sex hormones (EGAs) were submitted for further statistical analysis. From the data analysis, useful and valuable descriptive information about the natural steroid levels in bovines was obtained. In addition, an overview of the disturbance in plasma profiles of cattle treated with the oestrogenic compound was gathered.

1. Experimental

1.1. Samples

Serum samples were obtained from 72 Holstein cows that were between 24 months and 5 years in age, all from the same intensive dairy farm. Twenty-four cows were treated with an intramuscular injection of EB, consisting of 5 ml of a veterinary drug (Neonida N from Pfizer S.A., Madrid, Spain) containing 1250 I.U. of chorionic gonadotropin and 5 mg of EB, while 13 other cows were treated with an intravaginal device composed of a progesterone-releasing spiral (1.55 g of progesterone dispersed in an elastomeric silicone matrix) and a capsule containing 10 mg of EB (PRID® from CEVA Salud Animal, Barcelona, Spain). The experimental cows were fed a diet typically used in animal husbandry practices, and provided *ad libitum* access to water.

The administration of these hormonal preparations took place within a typical and real bovine reproductive control programme under the supervision of a veterinary surgeon. The estrogenic compounds were administrated before their total prohibition in October 2006. Blood samples from the animals treated with EB and EB combined with progesterone were collected on days 3 and 6, respectively, after a single-dose treatment. Thirty-five untreated animals were used as a control group (so-called non-treated or C).