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Excretion profile of boldenone in urine of veal calves fed two different milk replacers

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Abstract

The residue profiles of 17α-/17β-boldenone conjugated (17α/β-Bol) and ADD were investigated by liquid chromatography–tandem mass spectrometry (LC–MS/MS) in urine of male veal calves fed two commercial milk replacers, with different content of cholesterol and phytosterols. The urine samples were collected within 4 h after feeding and further from all the animals. Detectable amounts of 17α-Bol conjugated were measured in urine collected from all calves, but the concentrations of 17α-Bol were higher in urine from calves receiving the milk replacer with the greater amount of phytosterols. During the whole experiment, 17β-Bol and ADD were never detected in urine samples collected.

Keywords: Anabolic hormones; Alpha and beta boldenone metabolism; Milk replacer; Veal calves; Urine; Phytosterols; Liquid chromatography–tandem mass spectrometry

1. Introduction

Boldenone (androsta-1,4-dien-17β-ol-3-one or 17β-boldenone—17β-Bol) is an androgenic steroid of synthetic origin [1,2] obtained from dehydrogenisation of testosterone [3], endowed of potent anabolic properties. Because of its favourable effects on the animals' growth and feed conversion for meat production [4,5] it is illegally used for treatment of cattle and in racehorses and athletes to improve sport performance. Thus, 17β-Bol has been included between the 2005 WADA (World Anti-Doping Agency) list of banned substances [6].

The presence of boldenone residues, as for other anabolic agents such as steroidal androgynous hormones, is forbidden in live animals and their products under directives 96/22/EC and 03/74/EC [7,8] to avoid consumer exposure to unforeseeable risks from the intake of hormone residues and their metabolites. As for the other androgenic steroids, boldenone is classified by the International Agency for Research on Cancer (IARC) as a probable human carcinogen, with an index of carcinogenicity higher than those of other androgens, such as nandrolone, stanozolol, testosterone and clostebol [9]. It is also well known the role of 17α-boldenone (androsta-1,4-dien-17α-ol-3-one, 17α-Bol—the boldenone epimer) in the development of human prostate carcinomas implanted in mice [10].

Boldenone is physiological in a number of animal species, such as horses [11] and swine [12]. Presently, there is a scientific debate on the natural presence of 17α-Bol in bovine animals. Natural hormones and prohormones are frequently used for illegal treatments, as their exogenous origin cannot be easily demonstrable. The metabolic fate of steroidal hormones foreign to the animal body follows the pathway of the naturally occurring compounds, either after oral or parenteral administration [13].

A prohormone is a precursor, usually with minimal hormonal effect by itself, transformed into an active hormone by metabolic reactions. It has been demonstrated that androstadienedione (androsta-1,4-diene-3,17-dione or ADD) is the prohormone of 17α-Bol and 17β-Bol [14,15] in the bovine. Despite it is known that the oral administration of hormones used in cattle

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fattening is increasing [16], studies on experimental treatment of veal calves with 17β-Bol and ADD mainly concerned intra-muscular injections [5,17]. ADD has been identified as both an *in vitro* and *in vivo* metabolite, and it occurs in urines of veal calves treated with 17β-Bol [18] and when 17β-Bol is incubated with microsomes from calf liver [5]. Exposure of the invertebrate *Neomysis integer* (used as an alternative model to vertebrate animal experiments in metabolism studies) to ADD, led to the formation of androst-4-ene-3,17-dione (AED, the precursor of Testosterone) and 17β-Bol. Surprisingly, after exposure of the same organism to testosterone or stanozolol, small concentrations of 17β-Bol were observed, although the reproducibility of the experiments varied greatly [19].

Due to the polarity and the thermo-labile properties of metabolites in urine, the liquid chromatography combined with mass spectrometers (LC–MS/MS) has become the main analytical tool for trace level bioanalysis [25]. This technique allows the detection of very low concentrations without the need of derivatization as required for gas chromatography (GC/MS) [20].

Urinary excretion of 17α-Bol has been hypothesized as a consequence of endogenous production in cattle [4,21], likely related to feed quality, but no data are available on the urinary residues following the administration of feed with a high content of plant sterols.

To investigate "natural" in vivo elimination in cattle and to compare results with previous studies [22–24] the excretion profiles of 17α-, 17β-Bol and ADD in veal calves fed two commercial milk replacers characterized only for their content in cholesterol and phytosterols at different ratio, were studied.

2. Experimental

2.1. Animals

Fourteen Austrian Brown male veal calves, at the age of 60 days were settled in an authorized farm under controlled experimental conditions. The animals were housed for 1 month in ventilated stables in accordance with European Union animal wellness legislation and constantly monitored by the specialised staff and fed 2 months a commercial weaning diet. Appropriate measures were taken to avoid any kind of cross contamination between the animals and the access to the experimentation environments was restricted to the personnel involved in the study.

2.2. Chemicals and reagents

All solvents were HPLC or analytical grade and purchased from Riedel-de Haen (Seelze, Germany). Water was purified by MILLI-Q System (Millipore, Bedford, MA, USA). β-Glucuronidase/arylsulphatase (*Helix pomatia*) from Merck (Darmstadt, Germany) was used as supplied. 17α-Bol and 17β-Testosterone-d_2 were provided by RIVM (Bilthoven, The Netherlands). 17β-Bol was purchased from Riedel-de Haen (Seelze, Germany) and ADD from Steraloids (Newport, RI, USA).

2.3. Diet

After 2 months of housing, at the beginning of the experiment (day 0), the animals were split into two groups: seven calves received for further 2 months a commercial milk replacer usually employed in veal calf breeding practice (fed milk—FM), and seven were administered with another commercial milk replacer containing a higher percentage of phytosterols such sitosterol, campesterol, etc. (fed vegetal—FV). Table 1 reports the milk replacers' sterol composition: roughly a 30% of the cholesterol amount was replaced with several plant sterols, the main differences of sterol content are evidenced in italic.

The milk replacer FM contains 79 mg/100 g plant sterols as total amount with a cholesterol content of 87.4 mg/100 g, while in FV the phytosterols and cholesterol contents are 188 and 47.8 mg/100 g, respectively.

2.4. Sample collection and extraction

The first sample of urine was collected at day 0 when the diet was changed; the other samples were collected after roughly one month of FV and FM diet: at days 26, 27, 28, 36, 43, 50, 57, 61, 62, 63 and at day 65 (slaughter). Urines were collected (taking care to prevent faecal contamination) within 4 h from milk administration by using a clean container and waiting for spontaneous micturition: about 400–500 mL were collected and divided into 100 mL aliquots that were immediately stored in the dark at −20 °C. At slaughtering time urine was collected directly from the animal bladder and processed as above.

The urine extraction was performed as previously described by Draisci et al. [25], by using two aliquots of 2 mL each one. One aliquot was added with 6 mL of acetate buffer solution (ABS) 0.15 M, and spiked with the internal standard (I.S.) 17β-Testosterone-d_2. The mixture was added with β-glucuronidase/arylsulphatase enzyme solution (*Helix pomatia*), sonicated, incubated for 12 h at 37 °C, centrifuged and then purified by solid phase extraction (SPE) using a C_{18} cartridge (Baker C_{18}, 500 mg, 3 mL). The cartridge was previously con-

Table 1
Milk replacers' sterol composition (mg/100 g)

Substance	Fed vegetal (FV)	Fed milk (FM)
Cholesterol	*47.8*	*87.4*
Brassicasterol	0.3	0.3
24-Methylencholesterol	0.2	0.7
Campesterol	*30.5*	*12.0*
Campestanol	1.8	1.6
Stigmasterol	<0.1	<0.1
Delta-7-campesterol	*29.0*	*8.1*
Delta-5,23-stigmastadienol	1.7	1.1
Delta-5,24-stigmastadienol	5.6	1.9
Clerosterol	2.2	1.1
Beta-sitosterol	*103.2*	*44.9*
Sitostanol	3.0	2.0
Delta-5-avenasterol	6.4	3.3
Delta-5,23-avenasterol	2.6	1.4
Delta-7-stigmastenol	2.2	0.3
Total (phytosterols)	∼188	∼79