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Boldenone, Boldione, and Milk Replacers in the Diet of Veal Calves: The Effects of Phytosterol Content on the Urinary Excretion of Boldenone Metabolites

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Twenty-six veal calves were split into two groups and fed two milk replacers with a different content of phytosterols for 26 days; then, 14 calves (7 animals from each diet) were kept as controls and 12 calves (6 per diet) received daily, per os, a combination of 17β-boldenone (17β-Bol) and androsta-1,4-dien-3,17-dione (ADD) for 38 days. The urinary elimination of 17α-/17β-boldenone conjugates (17α/β-Bol) and androsta-1,4-dien-3,17-dione (ADD) was followed by liquid chromatography–tandem mass spectrometry from all of the animals until slaughtering. In urine from treated animals, 17α-Bol concentrations, despite a great variability, were greater than 17β-Bol, both detected always as conjugates. At days 1, 2, and 3, the mean urine concentration of 17α-Bol was higher than 12 ng/mL. A remarkable decrease was observed during the following days, but the 17α-Bol concentration was still higher than the attention level of 2 ng/mL in 58% of the samples; the concentration of 17β-Bol was around the action level of 1 ng/mL; two days after treatment withdrawal, no 17β-Bol was detected in the urine. In urine from control animals, the 17α-Bol concentration was strictly related to the phytosterol content of the diet, while, in urine from treated animals, the much higher 17α-Bol levels were not modified by the production from diet precursors. The results confirmed that a 17α-Bol level higher than 2 ng/mL should be considered as evidence of suspected illegal treatment and that the urinary excretion of 17β-Bol is due to exogenous administration of 17β-Bol. The discontinuous rate of elimination of both 17α- and 17β-Bol, despite the daily administration of 17β-Bol plus ADD, indicates the necessity for further research to detect other urinary boldenone metabolites to strength surveillance strategy.

KEYWORDS: Anabolic hormones; 17β- and 17α-boldenone; metabolism; veal calves; urine; liquid chromatography–tandem mass spectrometry

INTRODUCTION

Boldenone (androsta-1,4-dien-17β-ol-3-one) is a steroid which differs from testosterone only by one double bond at the 1-position and is frequently used as doping preparations in athletes and horses (*1*). Though this anabolic steroid is forbidden in EU countries (*2*, *3*) in calves and bulls bred for meat production, 17β-boldenone (17β-Bol) itself and its oxidized form, boldione (androsta-1,4-dien-3,17-dione or ADD), are illegally used as growth promoters, as they improve the growth and feed conversion in food producing animals (*4*).

In 2003, the results of several studies on 17β-Bol were reviewed to update the knowledge on the illegal use or the natural occurrence in cattle (*5*). The decision taken by the EU expert committee on the surveillance strategy of boldenone misuse in veal calves was that only discrimination between the conjugated and unconjugated forms of 17β-Bol in urine allows one to distinguish between endogenous occurrence and illegal use. Thus, the presence of 17β-Bol conjugates at any level was considered confirmation of illegal treatment because 17β-Bol conjugates were not detected as an endogenous substance in the urine of untreated veal calves. Conversely, sufficient scientific knowledge supported the presence of 17α-Bol in bovine urine from other sources than illegal treatment; thus, the presence of 17α-Bol conjugates should be considered a sign of suspect treatment only at a concentration higher than 2 ng/mL. The proposed minimum required performance limit (MRPL) of the analytical methods adopted for surveillance of 17β-Bol and 17α-Bol in bovine urine was 1 ng/mL (*6*).

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Several observations supported the neo formation of boldenone in fecal material of calves (*7*) and the natural occurrence (but not ubiquitous) of 17α-Bol in cattle urine (*8*, *9*). Recently, the possible endogenous status for this steroid was proposed as being related to feed quality, that is, the presence of phytosterols and other hormone precursors in animal feed (*7*).

In vitro studies proved the transformation of some phytosterols like β-sitosterol into substances with steroid structure and androgenic hormonal activity: in 1996 (*10*), it was reported that *Mycobacterium* sp. NRRLB3683 was capable of converting β-sitosterol to ADD and testosterone. A recent study (*11*) confirmed that also *Rhodococcus* strains by means of their pronounced cholesterol oxidase activity could convert β-sitosterol to testosterone. The phytosterols β-sitosterol and stigmastanol were reported to play a role in the production of ADD and boldenone also by means of *Neomysis integer* (*12*) and *Lucilia sericata* maggots (*13*) adopted as an alternative model in drug metabolism studies.

In the first part of this study, we confirmed the pivotal role of plant sterols in the excretion of detectable amounts of 17α-Bol conjugates in calf urine. Veal calves (14 animals) were fed two different milk replacers containing phytosterols at different amounts, the same used in the present study for 60 days (*14*). The highest concentrations of 17α-Bol conjugates in urine from calves receiving the milk replacer with the greatest percentage of plant sterols (e.g., β-sitosterol) confirmed the hypothesis that the presence of 17α-Bol is diet-related.

The present paper reports the further step of that study: to enhance the surveillance strategy, we compared in vivo the role of a 40 day treatment and the influence of dietary phytosterols on the urinary excretion rate of 17β-Bol and metabolites, in veal calves daily administered with 17β-Bol and ADD per os.

MATERIALS AND METHODS

Chemicals and Reagents. 17β-Bol was purchased from Riedel-de Haen (Seelze, Germany) and ADD from Steraloids (Newport, RI). 17α-Bol and 17β-testosterone-d_2 were provided by RIVM (Bilthoven, The Netherlands). All solvents were HPLC or analytical grade and purchased from Riedel-de Haen (Seelze, Germany). Water was purified by a Milli-Q system (Millipore, Bedford, MA). β-Glucuronidase/arylsulphatase (*Helix pomatia*) was purchased from Merck (Darmstadt, Germany).

Animals. Twenty-six Austrian Brown male veal calves (initial body weight (kg), 68.7 ± 0.13 SD) were settled and reared until the age of 120 days in an authorized farm under controlled experimental conditions and constantly monitored by the specialized staff. Appropriate measures were taken to avoid any kind of cross contamination between the animals, and access to the experimentation environments was restricted to the personnel involved in the study. The experimental plan was designed according to the guidelines of Italian law for care and use of experimental animals (DL 116/92), and the study was submitted for approval to the Italian Ministry of Health committee. The animals were housed in accordance with European Union animal wellness guidelines.

Diet. At the age of 120 days, the animals were split into two groups: one group (13 animals) received a commercial milk replacer usually employed in veal calf breeding practice, and the other (13 animals) was administered with another commercial milk replacer containing a higher percentage of phytosterols. The milk replacers' sterol composition was the same used in the previous study (*14*) and is reported in **Table 1**. One milk replacer (M) contained 79 mg/100 g of plant sterols as the total amount with a cholesterol content of 87.4 mg/100 g; in the other (V), the phytosterols and cholesterol contents were 188 mg/100 g and 47.8 mg/100 g, respectively.

Treatments. At the 146th day of age, 6 animals out of 13 from each diet were separated and grouped in two new treatment groups: calves treated milk (TM) and calves treated vegetal (TV). The treated animals received orally 0.9 mg of 17β-Bol and 0.1 mg of ADD previously dissolved in 5 mL of ethanol and added to 200 mL of reconstituted milk replacer. The administration was performed daily and immediately before morning feeding for 38 days. The seven animals in the former diet group remained as the control and received in the same way 5 mL of ethanol alone (fed milk, FM; fed vegetal, FV).

Table 1. Sterol Composition (mg/100 g) of the Two Milk Replacers

sterols	vegetal (FV – FM)	milk (TV – TM)
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
Δ-7-campesterol	29.0	8.1
Δ-5,23-stigmastadienol	1.7	1.1
Δ-5,24-stigmastadienol	5.6	1.9
clerosterol	2.2	1.1
β-sitosterol	103.2	44.9
sitostanol	3.0	2.0
Δ-5-avenasterol	6.4	3.3
Δ-5,23-avenasterol	2.6	1.4
Δ-7-stigmastenol	2.2	0.3
total (phytosterols)	**~188**	**~79**

Sample Collection. The first urine samples were collected 26 days before starting treatments, soon after the separation of the animals in the two groups receiving a different diet. Then, the urine samples were taken at days 1, 2, 3, 11, 18, 25, 32, 36, 37, and 38 from the beginning of treatment. Urine samples were collected also 2 days after the withdrawal of treatment when animals were slaughtered (day 40 - final body weight (kg), 245 ± 15 SD).

As reported (*14*), urines were collected within 4 h after treatment at spontaneous micturition; about 400–500 mL were collected and divided into 100 mL aliquots that were immediately stored in the dark at −20 °C.

Sample Extraction. The urine extraction was performed as previously described by Draisci et al. (*14*, *15*), by using two aliquots of 2 mL each. To detect the presence of free boldenone, one aliquot was extracted after the deconjugation process and the other without.

Liquid Chromatography–Tandem Mass Spectrometry Analysis. Analyses were carried out by using an LC system Perkin Elmer Series 200 Micro Pump (Perkin Elmer, U.S.A.) equipped with an API 3000 triple quadrupole mass spectrometer (AB Sciex Instruments, Foster City, CA). The analytes were monitored in multiple reaction monitoring (MRM) mode; the transition chosen for each compound and method performances are summarized in **Table 2** and reported in extenso in refs *14 and 15*.

Method Validation. The validation was performed in accordance with Commission Decision 2002/657/EC (*16*). An extensive description of the analytical method adopted by liquid chromatography–tandem mass spectrometry (LC–MS/MS) allowing the simultaneous detection of 17α-Bol, 17β-Bol, and ADD in bovine urine with good sensitivity and repeatability was already reported (*14*). In brief, to demonstrate that the method is suitable for the intended purpose, blank urine samples (2 mL) were spiked, daily, with 5 ng/mL of internal standard (IS) followed by mixtures of 17α-Bol, 17β-Bol, and ADD to obtain five concentration levels of 0.50–20.0, 0.20–5.0, and 0.99–50.8 ng/mL, respectively, and six replicate samples at each level. The series were analyzed in 3 different days over three analytical runs to evaluate repeatability and within-laboratory reproducibility (different conditions and operator). The recovery (internal standard corrected) was determined as the percentage between the mean concentration per analytical run and the nominal concentration. The coefficient of variation (CV) was used to report the inter-assay precision.

Peak area ratios of the analyte to IS were plotted versus hormone concentrations and a least-squares linear regression model was adopted to calculate matrix calibration curves, used to estimate the analyte amounts in spiked and incurred samples. A good linearity of calibration curves was confirmed for all of the anabolic compounds at all

Table 2. Precursor, Most Abundant Fragments, and Method Performances for the Steroids Analyzed in MRM Mode (*13, 14*)

analyte	precursor *m/z*	product *m/z*	CE (eV)	DP (V)	t_R (min)	CCα (ng/mL)	CCβ (ng/mL)
17β-Bol	287.4 $(M + H)^+$	121.1, 135.3, 173.1	21	23	5.8	0.15	0.40
17α-Bol	287.4 $(M + H)^+$	121.1, 135.3, 173.1	21	23	7.1	0.25	0.40
ADD	285.4 $(M + H)^+$	121.2, 147.3, 151.1	21	26	6.9	0.34	0.50
17βT-d_2[a]	291.5 $(M + H)^+$	99.1	27	35	7.2		

[a] 17βT-d_2 internal standard.

Table 3. Recovery, Within-Laboratory Reproducibility, and CV% for 17α-Bol, 17β-Bol, and ADD

analyte/parameter	spike level (ng/mL)	concentration found (ng/mL)	recovery (%)	within-laboratory reproducibility (RSD, $n = 18$)	CV %
17α-Bol	0.50	0.52	103.7	0.05	10.6
	1.00	1.02	102.4	0.03	3.1
	2.00	2.01	100.9	0.06	2.9
	10.00	9.96	99.6	0.15	1.5
	20.00	20.02	100.1	0.07	0.4
17β-Bol	0.20	0.20	100.9	0.01	7.2
	0.50	0.49	99.2	0.02	3.8
	1.01	1.01	100.5	0.02	2.6
	2.02	2.01	99.9	0.04	2.1
	5.05	5.05	100.0	0.02	0.3
ADD	0.99	0.95	95.8	0.12	12.4
	4.98	4.92	98.7	0.32	6.6
	10.15	10.18	100.2	0.26	2.5
	20.34	20.49	100.7	0.49	2.4
	50.83	50.77	99.9	0.63	1.2

concentrations checked, as proved by the correlation coefficients (*r*), all greater than 0.9980.

RESULTS

All three compounds were quantified at a concentration level lower than 1 ng/mL. The decision limit (CCα) was 0.15 ng/mL for 17β-Bol, 0.25 ng/mL for 17α-Bol, and 0.34 ng/mL for ADD. The detection capability (CCβ) was 0.40 ng/mL for 17α/17β-Bol and 0.50 ng/mL for ADD. For each analyte, the matrix calibration curve was evaluated in the range CCα, –50 ng/mL; the good linearity was proved by the correlation coefficients all greater than 0.998. In **Table 3** are reported the results obtained for accuracy, reproducibility, and variation coefficient for each of the three compounds analyzed. In **Figure 1** are shown the extracted ion currents (XIC) in MRM mode of 17α/β-Bol, ADD, and 17β-testosterone-d_2 added to a blank urine at the highest concentrations used in the matrix calibration curve (20.0, 5.0, 50.8, and 5 ng/mL).

In the urine samples collected from treated calves and before the beginning of treatments and in samples from control calves, no 17β-Bol was detected. 17α-Bol was detected in several urine samples before treatment and from the control group at a mean value lower than 1 ng/mL, and in all positive samples from treated animals, 17α-Bol and 17β-Bol were found at different concentrations. All were found only after deconjugation with β-glucuronidase/arylsulfatase (**Figures 2** and **3**); free 17α-Bol was detected in 8% of the urine samples extracted without the deconjugation process; free 17β-Bol at 3.7 ng/mL was detected only in one sample from the TV group.

The amount of 17α-Bol conjugates detected in urine from control calves (FV and FM groups) was closely related to the amount of β-sitosterols in milk replacers: the replacer with a higher amount of phytosterols, double that of the other diet M, caused the greater excretion of 17α-Bol conjugates (FV > FM) (**Figure 4**).

Figures 5 and **6** show the concentrations of 17α-Bol and 17β-Bol conjugates in urine samples collected from TV and TM calves during the treatment (mean values ± SD). The mean concentrations of 17α-Bol in urine collected at day 1 (within 4 h after treatment) from both TM and TV animals were 36.30 and 15.98 ng/mL, respectively; after days 2 and 3 of treatment, the elimination rate of 17α-Bol almost halved in urine from TM animals; during the following days, a slower decrease in urine of both groups was observed (**Figure 5**). The overall amount of 17α-Bol excreted, although varying greatly, was by far greater than that excreted before the treatment or by control animals and by far greater than that of 17β-Bol.

Levels of 17α-Bol higher than 2 ng/mL (DG SANCO recommended attention level) (*6*) were present in 57.1 and 58.6% of the samples from TV and TM animals, respectively (**Figure 7**). However, in samples collected during the first 3 days of treatment, the level of 17α-Bol was higher than 2 ng/mL, in all samples from the seven TM calves (100%) and in 83% of the TV group.

At slaughtering time (2 days after treatment withdrawal), different concentrations of 17α-Bol were recorded from the two groups: for TM calves, the mean daily value was 0.45, and for TV, it was 2.15 ng/mL.

During treatment, the excretion of 17β-Bol varied considerably: the mean daily concentrations of 17β-Bol were in the range 0.15–1.3 ng/mL. The maximum was reached at days 32 and 37 only in samples from TV calves (**Figure 6**).

Concentrations greater than a CCα value of 0.15 ng/mL were detected in samples from TV calves, 45 animals out of 56 (80.4% positive), and from TM ones, 49 out of 58 (84.5%). The percentage of positive samples with respect to the total amount was 21.4 % of the TV group and 12% of the TM group (**Figure 8**). In urine samples collected directly from the bladder at the slaughter house, 2 days after treatment withdrawal, no 17β-Bol was detected.

The presence of ADD was detected only a few times, at a level around the CCα. There was only one exception: one sample from the TV group, collected the first day of treatment, contained ADD at 1.5 ng/mL and free and conjugated 17β-Bol at 3.7 and 2.2 ng/mL, respectively.

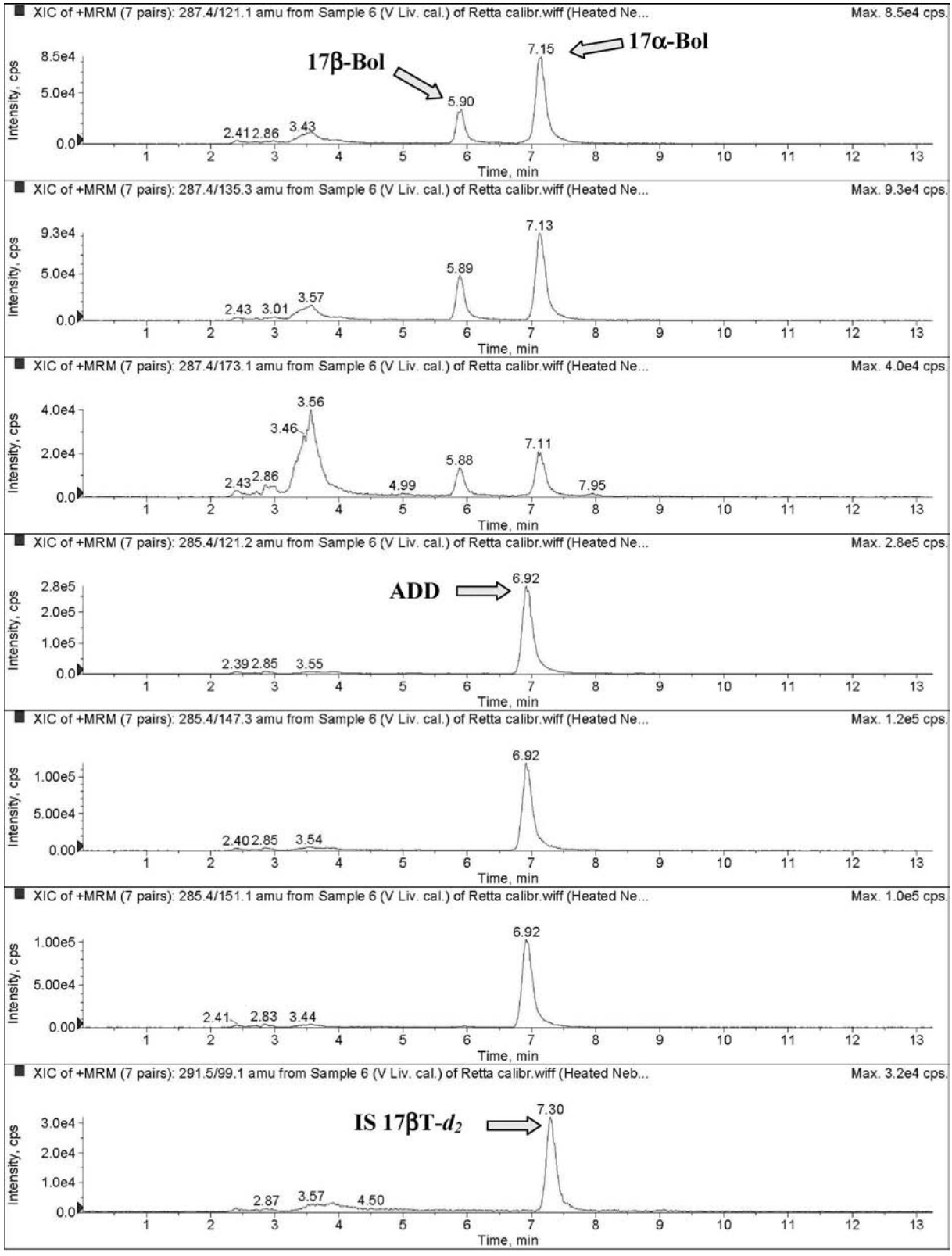

Figure 1. Extracted ion currents (XIC) in MRM mode of 17α/β-Bol, ADD, and 17β-testosterone-d_2 added to a blank urine at the highest concentrations used in the matrix calibration curve (20.0, 5.0, 50.8, and 5 ng/mL).

DISCUSSION

To observe the influence of phytosterols contained in milk replacer for veal calves on the excretion rate of boldenone metabolites, the amounts of 17α-, 17β-Bol, and ADD were measured in urine from treated and untreated calves during a 40 day experiment. The combination of 17β-Bol and ADD was administered orally via milk diet and given in a 9:1 ratio. The ratio adopted was similar to that illegally used in breeding practice, as indicated by the National Surveillance Authority "Carabinieri–Nucleo Antisofisticazioni e Sanità" after several constraints of farm preparation containing mixtures of boldenone and ADD and positive urine samples collected at veal farms.

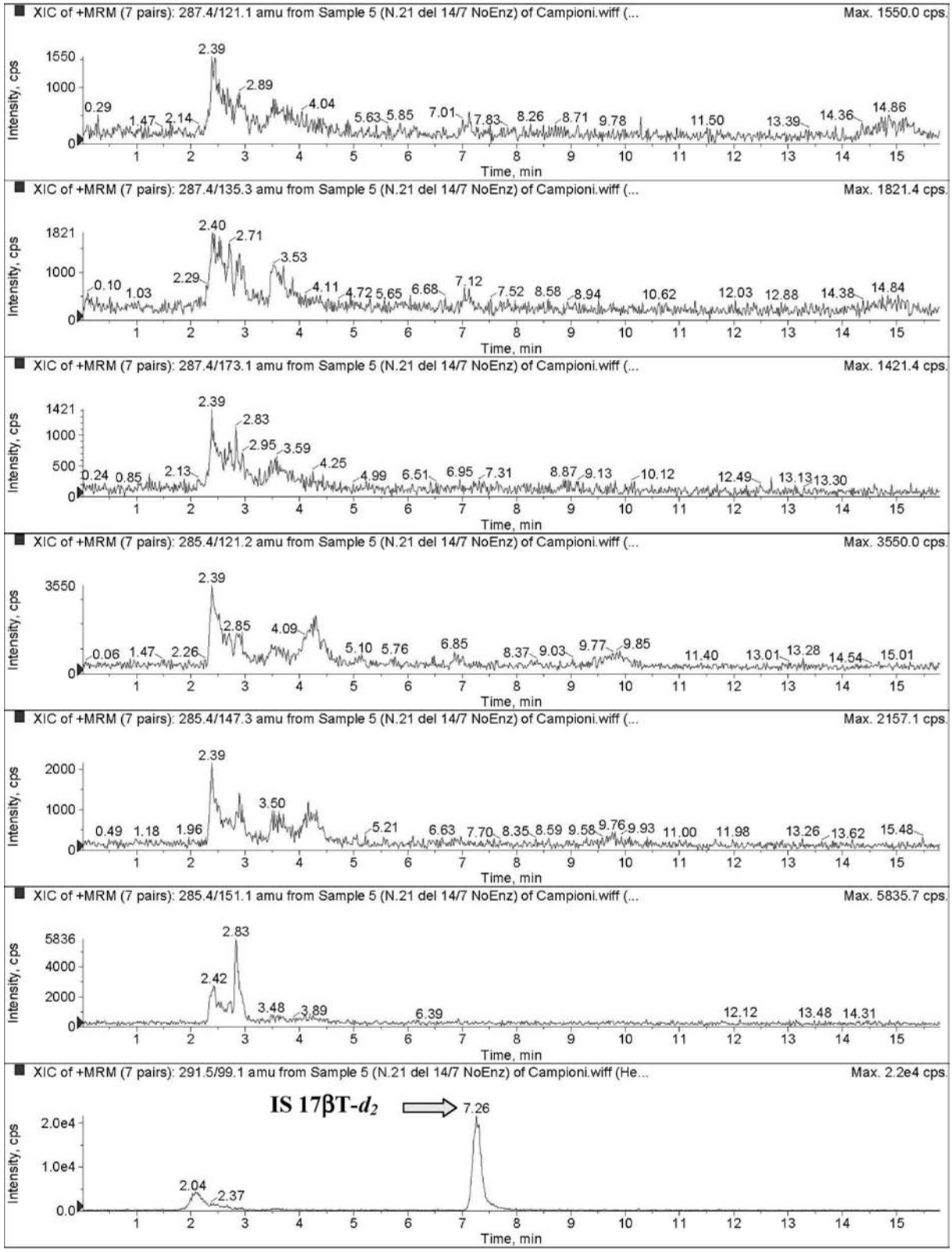

Figure 2. Extracted ion currents (XIC) in MRM mode of 17α/β-Bol, ADD, and 17β-testosterone-d_2 of an incurred urine sample before enzymatic deconjugation.

The high variability observed in the excretion of 17α- and 17β-Bol could be ascribed to the choice of sampling (waiting for spontaneous micturition and collecting the variable amount of urine produced) and to the lack of normalization of the data, but we applied the same procedure commonly adopted during surveillance practice.

In the absence of any exogenous 17β-Bol administration, 17α-Bol conjugates was detected at higher concentrations in urine from calves receiving the milk replacer with the greater amount of β-sitosterol (*14*), but levels in urine were below the recommended limit of DG SANCO. Thus, the theory that part of the urinary 17α-Bol content could be diet-related and that

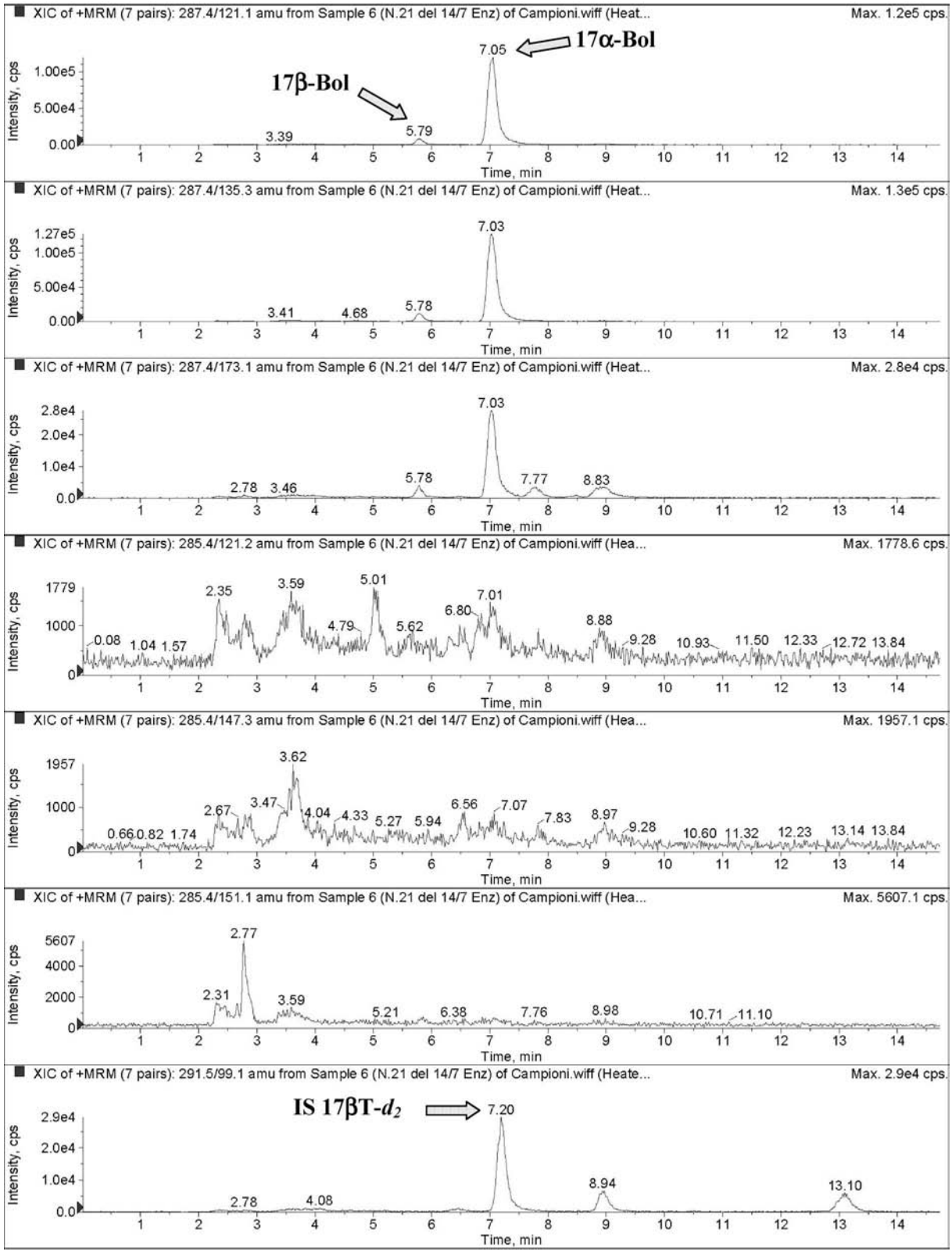

Figure 3. Extracted ion currents (XIC) in MRM mode of 17α/β-Bol, ADD, and 17β-testosterone-d_2 of an incurred urine sample after the enzymatic deconjugation. 17α-Bol was found at 33.7 ng/mL and 17β-Bol at 1.45 ng/mL. IS was added at 5 ng/mL.

β-sitosterol could play a role in the production of ADD as a precursor and then in the 17α-Bol conjugates excreted in urine is confirmed.

The amount of 17α-Bol excreted a few hours after the first treatment in urine from treated animals confirmed that 17β-Bol and ADD were rapidly absorbed per os and promptly metabolized in 17α-Bol, the major in vivo metabolite (*6*, *17*). The overall amount of 17α-Bol excreted during the treatment was by far higher than that excreted in the absence of treatment; this could be due to the transformation of the administered combination of 17β-Bol plus ADD into the inactive α-epimer as the main metabolite. The metabolic pathway supposed is that

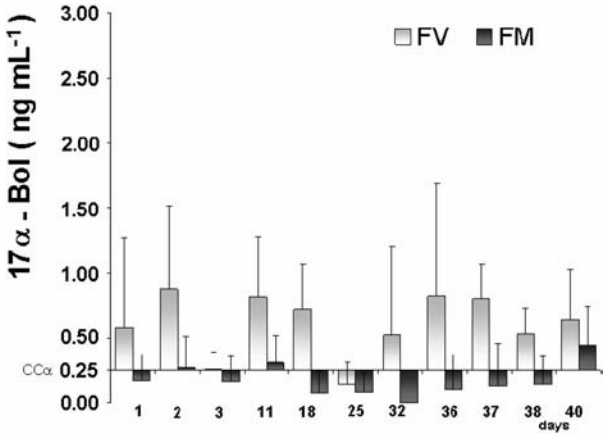

Figure 4. Daily excretion profile of 17α-Bol conjugates in urines from FV and FM calves. Values are reported as mean ± SD (negative values are adopted to indicate that only a few urine samples, out of the seven collected that day, were below CCα).

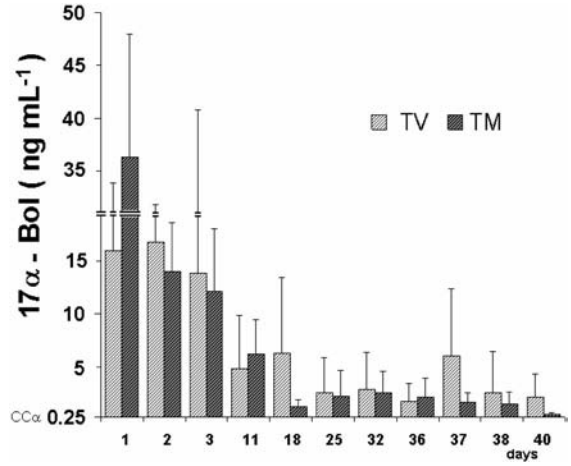

Figure 5. Daily excretion profile of 17α-Bol conjugates (ng/mL) detected in urine from TV and TM calves during the 38 day treatment and at slaughter time (40th day). Values are reported as the mean ± SD of all urine sample collected the same day.

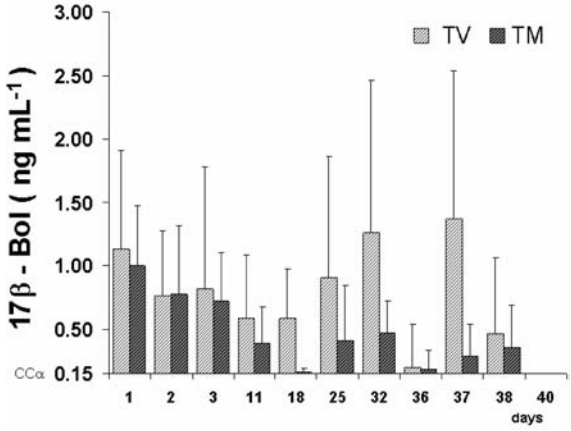

Figure 6. Daily excretion profile of 17β-Bol conjugates (ng/mL) detected in urine from TV and TM calves during the 38 day treatment and at slaughter time (40th day). Values are reported as the mean ± SD of all urine samples collected the same day.

17β-Bol undergoes an oxidation by 17β-hydroxysteroid oxido-reductase leading to ADD and then ADD is reduced by the very active 17α-hydroxysteroid oxido-reductase to give 17α-Bol.

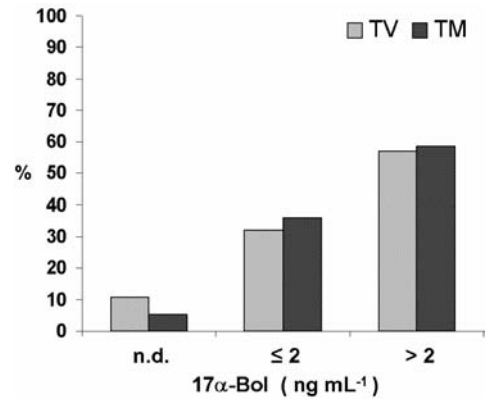

Figure 7. Distribution of 17α-Bol conjugate concentrations below or above the DG SANCO recommended limit (2 ng/mL) in urine samples from TV and TM calves.

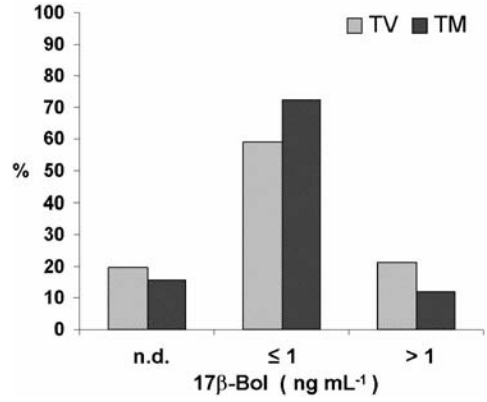

Figure 8. Distribution of 17β-Bol conjugate concentrations below or above the DG SANCO recommended limit (1 ng/mL) in urine samples from TV and TM calves.

The amount of 17α-Bol in urine, always higher than that of 17β-Bol, suggested, as it occurs for testosterone and nortestosterone (*18*), that the rate of interconversion of 17β-Bol to 17α-Bol occurs faster (much faster than the reverse reaction), and also that the conjugation rate of 17α-Bol could be faster than that of 17β-Bol.

During the treatment, with the exception of the first day, 17α-Bol concentrations in the urine of treated TM and TV calves were of the same magnitude, as the greater excretion rate after treatment masked the differences of 17α-Bol excretion in urine from FV and FM calves (**Figure 5**).

Our results confirm that the exogenous daily administration at low dosage of 17β-Bol and ADD (0.9 + 0.1 mg/head, respectively) causes the excretion of 17α-Bol at amounts much higher than that caused by the phytosterols occurring in the diet. Thus, detecting 17α-Bol above 2 ng/mL could be attributed to 17β-Bol exogenous administration rather than a possible endogenous or diet-related origin.

The presence of 17β-Bol conjugates in urines was recorded only after 17β-Bol exogenous administration and confirms that this conjugated metabolite is the marker of illegal use (*19*).

In a recent paper by Fidani et al. (*20*), it was reported that bacteria present in equine urine samples, as a consequence of poor storage conditions, may cause degradation of 17β-OH steroids. The stereoselective reaction occurred in β-epimers only, that is, testosterone, nandrolone, trenbolone, and boldenone, and not in the α-epimers such as 17α-Bol and epitestosterone. Thus, in the presence of bacterial activity, the β-steroids were transformed into their respective ketones, and the presence of

ketones could be used as a marker of microbiological contamination.

On the basis of data reported by Fidani et al. (*20*) and the analytical limit of detection for ADD, we can not exclude that low levels of microbiological contamination could prevent the detection of 17β-Bol, but this would be restricted to a very low amount in a few samples.

The method of sample collection, storage conditions, and analytical procedure adopted for urine specimens of this study reduced considerably the number of samples containing oxidated ketones: ADD was found at a concentration around CCα in less than 10% of the analyzed samples. Only one sample revealed the presence of ADD at high concentration; this could be a consequence of a partial oxidation of the high amount of free 17β-Bol by microbiological contamination.

In our experiment, the low dosage chosen and the per os administration allowed detection only during the treatment. Two days after the suspension of treatments, no 17β-Bol was detected, while it is reported that the 17β-Bol elimination, after intramuscular injection of 17β-Bol undecanoate, was detectable until 20 days after treatment withdrawal (*21*). Many variables could influence the number of incurred urine samples: the different route of administration, oral versus intramuscular; the pharmaceutical preparation, milk solution versus oily injectable solution; the compound chosen as free hormone versus the esterified one; and the last dosage.

Despite the daily administration, the decrease in the 17α-Bol concentration after the first week was remarkable (**Figure 5**), and with the exception of TV calves at days 25, 32, and 37, the 17β-Bol urinary levels (**Figure 6**) showed a decreasing trend during treatment. The rapid decrease of urinary concentration could be due to the involvement of liver enzymes other than hydroxysteroid oxido-reductases, that is, cytochrome P450 dependent hydroxylases (known as mixed function oxygenases or MFO), catalyzing the transformation of 17β-Bol and ADD into other metabolites.

The in vitro metabolism of 17β-Bol and ADD confirmed that several hydroxylated derivatives could be produced in bovine liver microsomes, among those 6β-hydroxy-17β-Bol and 6β-hydroxy-ADD (*4*, *22*). Also, 17α-Bol incubated with bovine liver microsomes and the NADPH generating system led to ADD and to a hydroxylated metabolite identified as 6β-hydroxy-17α-Bol (*23*).

Ongoing in vitro studies on the MFO of livers collected from the animals that underwent the treatment revealed a fable induction only on a few P450 isoforms (unpublished data) in livers from the TM veal calves, the group which showed the greater decrease of conjugate excretion versus time.

The repeated oral administration, at a fixed dosage for more than 1 week, could be responsible for the decreased excretion of conjugated 17α- and 17β-Bol, as the conjugation of other hydroxy-boldenone derivatives could take place.

As defined by Le Bizec (*24*), the ideal marker should correspond to the following definition: always detectable whatever the administered (injected) form and never observable in the so-called "endogenous" case. Currently, several studies were devoted to detect specific metabolites (produced either from phase I or phase II reactions) as the marker of treatment, and the same authors support 17β-Bol sulfate as a good candidate for confirmatory strategy to unambiguously distinguish treated from nontreated animals.

It is likely that the detection in bovine urine of other hydroxyl derivatives other than 6β-hydroxy-17α-Bol and 6β-hydroxy-17β-Bol, already identified as 17β-Bol metabolites in vitro and in vivo (*23*, *25*) and recently adopted as screening metabolites (*26*), could help to strengthen surveillance strategy.

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