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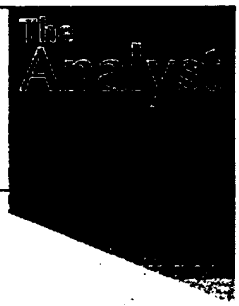
Identification of some important metabolites of boldenone in urine and feces of cattle by gas chromatography-mass spectrometry†



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17α-Boldenone (17α-BOL) and/or 17β-boldenone (17β-BOL) appear occasionally in fecal matter of cattle. In addition to 17α-BOL, a whole array of boldenone related substances can be found in the same samples. *In vitro* experiments with microsomal liver preparations and isolated hepatocytes combined with the excretion profiles found in urine and feces samples of *in vivo* experiments made it possible to identify several metabolites of 17β-BOL in 17β-BOL positive feces samples. In one animal treated with 17β-BOL, no 17β-BOL or its metabolites were present before treatment and most of these compounds disappeared gradually in time after the treatment was stopped. It is not clear what the origin is of 17α-BOL and boldenone metabolites in samples screened routinely for the abuse of anabolic steroids and considered to be 'negative' because of the absence of 17β-BOL since other workers showed some evidence that 17α-BOL can be of endogenous origin. However, in our hands, most of these 17α-BOL positive samples, obtained during routinely performed screenings of cattle, contained large amounts of Δ^4-androstene-3,17-dione (AED), which normally is absent from routinely screened negative samples. Furthermore, AED was absent in all samples obtained from the animals treated with 17β-BOL. We have no direct evidence that 17α-BOL or 17β-BOL is of endogenous origin.

1. Introduction

17α-Boldenone or androsta-1,4-dien-17α-ol-3-one (17α-BOL) can occasionally be found in feces from cattle screened for the presence of illegal growth promoters. Sometimes, a trace of 17β-boldenone or androsta-1-4-dien-17β-ol-3-one (17β-BOL) can be found in the same samples. 17α-BOL is considered to be the main metabolite of 17β-BOL in human and equine urine.[1,2] 17β-BOL improves the growth and food conversion of cattle and therefore can be abused for more efficient meat production. From the analysis of application sites there is no evidence that 17β-BOL or one of its esters is used in Belgium. To our knowledge, no abuse of 17β-BOL or one of its esters has been reported in Belgium.

This provokes a series of questions about the possible origin of 17α-BOL found in field samples, the metabolic conversion of 17β-BOL in cattle and the metabolites that can be found in urine and feces after administration of 17β-BOL to cattle. The detection of 17α-BOL would become meaningful as evidence for the abuse of 17β-BOL if at the same time additional metabolites of 17β-BOL could be identified. Studies have been performed on the excretion of 17β-BOL and/or its metabolites in human and equine urine, but so far no information is available on excretion in feces.[1,2] The excretion of boldenone metabolites in horse urine is different from the excretion profile in humans.[2–4] The main metabolic pathways in horses involve 17α-epimerisation to 17α-BOL and 16-hydroxylations. In humans, however, seven metabolites have been reported, including oxidation of 17β-hydroxy to 17-oxo, 6β-hydroxylation and reduction of the Δ^4 double bond and/or 3-oxo function.[1] This interspecies variation indicates the importance of studying the metabolism in cattle. A study on the metabolism *in vivo* of 17β-BOL in cattle performed previously was limited to the excretion of 17β-BOL and /or 17α-BOL in urine.[5] There have been no reports on other possible metabolites of 17β-BOL and/or their excretion in feces.

In this study, microsomes and isolated hepatocytes prepared from liver tissue were used to identify the most important metabolic pathways in cattle. In addition, we looked for possible metabolites of 17β-BOL in urine samples collected from one animal treated with 17β-boldenone undecanoate (obtained from R. Schilt, RIKILT-DLO, Wageningen, The Netherlands) and one animal treated with 17β-BOL (obtained from H. De Brabander, State University of Ghent, Faculty of Veterinary Medicine, Ghent, Belgium). In the latter animal we looked also for breakdown products in feces.

2. Experimental

Materials

Microsomes were prepared from livers of two 36-week-old bulls. Hepatocytes were isolated from the liver of a Friesian–Holstein bull about 1 year old.

Urine samples from animals treated with 17β-boldenone undecanoate were obtained from R. Schilt. We did not obtain

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any samples from this animal before treatment (= control). Urine and feces samples of an animal (625 kg) injected intramuscularly with 700 mg of 17β-BOL were provided by H. De Brabander. From this animal we obtained samples before, during and after treatment.

All reagents and solvents were of analytical-reagent grade. Milli-Q water was prepared with a Waters Milli RO water purification system (Millipore, Bedford, MA, USA). All anabolic steroids were obtained from the Belgian national reference laboratory (Wetenschappelijk Instituut voor Volksgezondheid–Louis Pasteur, Brussels, Belgium). 5α-Androst-1-ene-3,17-dione was obtained from Sigma (St. Louis, MO, USA) and 17α-BOL from TNO (Zeist, The Netherlands). All reagents used for the isolations and incubations *in vitro* were obtained from Sigma. All reagents required for the synthesis of metabolites were purchased from Aldrich (Milwaukee, WI, USA).

In vitro experiments

Microsomes were prepared from liver and incubated with steroids as described earlier.[6] Hepatocytes were isolated according to the method of Seglen[7] modified by Van't Klooster *et al.*[8] Cells were cultured at a density of 4×10^6 cells per 60 mm culture dish (Greiner, Alphen a/d Rijn, The Netherlands) in 4 ml of Williams E supplemented with 4% newborn calf serum, 1.67 mmol l^{-1} glutamine, 50 μg ml^{-1} gentamicin sulfate, 1 μmol l^{-1} hydrocortisone, 1 μmol l^{-1} insulin, 0.5 mmol l^{-1} $CaCl_2$ and 0.5 mmol l^{-1} $MgCl_2$. Cells were incubated for 4 h in a humidified atmosphere of air (95%) and CO_2 (5%) at 37 °C. The medium was then replaced with medium without serum, $CaCl_2$ and $MgCl_2$. After an incubation of 20 h, the hepatocytes were either incubated with 100 μmol l^{-1} boldenone for 6 and 24 h or with 10 μmol l^{-1} of boldenone for 24 h. The steroids were dissolved in MeOH. The final concentration of methanol was 0.1% in the incubation medium. After incubation, the samples were frozen at −20 °C until GC-MS analysis was performed.

Sample preparation

The clean-up of urine and feces sample was carried out as described previously.[9,10] Boldenone and its metabolites were isolated from the microsomal incubation mixtures by liquid–liquid extraction.[6] For the hepatocytes, 2 ml of the incubation medium were adjusted to pH 5.2 with acetate buffer (0.2 mol l^{-1}, pH 4.8). A 50 μl volume of Succus *Helix pomatia* was added to the mixture, which was then incubated overnight at 37 °C. The hydrolysed medium was applied to a Chem-Elut column (Varian, Harbor City, CA, USA). Steroids were eluted from the column with 5 ml of *tert*-butyl methyl ether and 5 ml of chloroform.

HPLC fractionation of extracts

The HPLC system used was equipped with a Model 231 high-pressure pump (Spectra Physics, San Jose, CA, USA) with a Rheodyne (Cotati, CA, USA) Model 7010 injection valve, a Model 2151 variable wavelength monitor (LKB, Bromma, Sweden) operating at 244 nm, a Model 202 fraction collector (Gilson, Worthington, OH, USA) and a Chromatopack CR-IB recorder (Shimadzu, Kyoto, Japan). The HPLC column was a 250 × 10 mm id stainless-steel tube packed with 5 μm silica (ODS Ultrasphere, Beckman, Fullerton, CA, USA). Methanol–water was used as the mobile phase for gradient elution at a rate of 2.5 ml min^{-1}. The collector was operated in the time mode. Ten fractions were collected with time windows relative to naphthalene, used as an internal standard, ranging from 0.20 to 0.32 (fraction 0), 0.32 to 0.42 (fraction I), 0.42 to 0.54 (fraction II), 0.54 to 0.65 (fraction III), 0.65 to 0.75 (fraction IV), 0.75 to 0.89 (fraction V), 0.89 to 1.03 (fraction VI), 1.03 to 1.15 (fraction VII) and 1.15 to 1.27 (fraction VIII). All fractions were dried separately under a stream of nitrogen at 60 °C and the residue was dissolved in 500 μl of methanol. A 250 μl volume was placed in a GC-MS vial. 5α-Androstan-3α-ol-17-one was added as internal standard to fractions I–V and 5α-estrane-3β,17α-diol to fractions VI–VIII. A 25 μl volume of a 5 ng μl^{-1} solution of the internal standard in methanol was added. The vials were dried under nitrogen at 60 °C and derivatised to ethoxime-trimethylsilyl derivatives. All fractions obtained were injected separately.

Gas chromatography-mass spectrometry

To produce ethoxime-trimethylsilyl (EO-TMS) derivatives, the dry residues were dissolved in 100 μl of ethoxylamine solution and heated for 90 min at 80 °C. Subsequently, the excess of reagent was evaporated at 60 °C under a stream of nitrogen. To the dry residue, 25 μl of silylating mixture (TBT) were added and the sample was incubated overnight at 80 °C.[9] Trimethylsilyl derivatives were prepared as described previously.[1] The GC-MS analyses were performed on a Finnigan (San Jose, CA, USA) GCQ system. The GC column used was a DB-5 MS fused silica column (30 m × 0.32 mm id) with a 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). Temperature settings were as follows: injector 260 °C; transfer line 275 °C; ion source 200 °C; and oven program, initial temperature 50 °C for 20 s, increased from 50 to 190 °C at 50 °C min^{-1} and from 190 to 320 °C at 4.6 °C min^{-1}, with a hold at 320 °C for 4 min. The carrier gas was helium at a gas velocity of 60 cm s^{-1}.

GC-MS data handling

For positive identification of a compound, the same criteria as described earlier[9] were used. Semi-quantitative results were obtained by measuring the peak area ratio of a selected fragment ion relative to the area of one particular fragment ion of the internal standard (5 ng μl^{-1}): *m/z* 360 of 5α-androstan-3α-ol-17-one and *m/z* 242 of 5α-estrane-3β,17α-diol. To calibrate the instrument, direct control samples of 10, 5, 2, 1 and 0.5 ng μl^{-1} were injected. Each analyte was quantified in triplicate using three different fragment ions.

Quality control of the clean-up of excreta

The overall extraction recoveries were checked for each batch of 10 samples prepared by processing a blank sample spiked with 17β-boldenone, androsta-1,4-diene-3,17-dione, 5α-androst-1-ene-3,17-dione, equilenin and some other reference standards of steroids, all at the level of 2 ng ml^{-1} for urine and 10 ng g^{-1} for feces, as described earlier.[9] An internal control for each urine and feces sample of the animal experiments and the routine feces samples was provided by the addition of equilenin at 2 ng ml^{-1} for urine samples and at 10 ng g^{-1} for feces samples. This also allowed us to correct the calculations of the excretion levels of 17β-boldenone, 17α-boldenone, androsta = 1,4-diene-3,17-dione and 5α-androst-1-ene-3,17-dione for the differences in recoveries between the samples.

3. Results and discussion

The liver is the main organ responsible for a wide variety of metabolic reactions of xenobiotics. Two different types of liver

preparations were used to assess the hepatic biotransformation of 17β-BOL. Microsomes and monolayer cultures of isolated hepatocytes were used to allow a quick identification of important metabolites on the one hand and to reduce the need for expensive and labor-intensive animal studies on the other. Since phase I oxidative enzymes are almost exclusively localized in the endoplasmic reticulum, it is appropriate to use microsomes to study the oxidations of the steroid nucleus in a low matrix environment. The phase I oxidative biotransformation products formed are indicative of the orientation of the screening methods to be used for the analysis of urine and feces samples. However, primary cultures of hepatocytes isolated from livers of slaughtered cattle allow a more accurate quantitative study of the different metabolic routes. Moreover, the formation of metabolites can be monitored directly within the system. To evaluate the *in vitro* assays and their relationship to the *in vivo* situation, the metabolism of 4-chlorotestosterne (CLT) was also monitored.

In vitro results

The oxidation of 17β-BOL to androsta-1,4-diene-3,17-dione (ADD) was found to be the most prominent *in vitro* metabolite in microsomal incubations. Hydroxylation at the allylic position 6 led to some minor metabolites of both 17β-BOL and ADD. It was found that 36% of CLT, extensively metabolized *in vivo*, was metabolized *in vitro* while the same batches of microsomes metabolized only 4% of 17β-BOL, which, like CLT, is also extensively metabolized *in vivo*.

Incubations with isolated hepatocytes led to large amounts of 6-hydroxylated metabolites of both 17β-BOL and ADD, as shown in Fig. 1 The ion chromatograms show double peaks for ADD, 17α-BOL and 17β-BOL. *Syn* and *anti* isomers are formed with the non-sterically hindered keto groups. More details of this isomer formation is described in the literature.[11–13] In addition to the 6-hydroxylated metabolites, some other hydroxylated metabolites, which were only identified tentatively, were detected. The 17α-epimerisation product, 17α-BOL, was found to a minor extent only. ADD and 17α-BOL were identified by comparison with available compounds. 6-Hydroxy-17β-boldenone (6-HO-BOL) and 6-hydroxyandrosta-1,4-diene-3,17-dione (6-HO-ADD) were synthesized according to a method described earlier.[1] Their mass spectra are shown in Fig. 2 and the necessary data for identification are given in Table 1. Both CLT and BOL were extensively metabolised by the monolayer cultures of isolated hepatocytes. CIT was already complety metabolised from the incubation medium after 6 h of incubation and boldenone to the extent of 88% after 24 h of incubation.

Furthermore, it was observed in primary cultures of liver cells that a limited amount only of the Δ^4 and 3-oxo functions of 17β-BOL and/or ADD are reduced. As an example of partial identification, two spectra are shown in Fig. 3. The characterization of some other reduced compounds is the subject of further investigation. However, evidence for the presence of a Δ^1 double bond was found by oxidizing all hydroxy groups to ketones and subsequent identification of 5β-androst-1-ene-3,17-dione (5β-AED) as the main reaction product with pyridinium chlorochromate.[14] Nevertheless, this still leaves several possibilities open: 5ξ-androst-1-en-3ξ-ol-17-one or 5ξ-androst-1-en-3-one-17ξ-ol (5ξ-AE). 5β-AED itself, reported as a metabolite of β-BOL in human urine, was not observed after

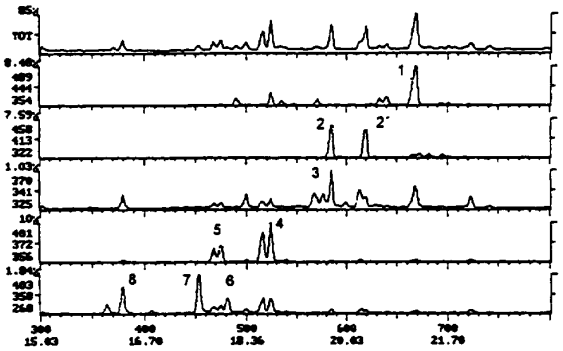

Fig. 1 Total ion current chromatogram and ion chromatograms (sum of the three most important diagnostic ions of the EO-TMS derivatives are shown on the ordinate) of 17β-BOL and its most prominent *in vitro* metabolites after a 24 h incubation of 17β-BOL with isolated hepatocytes: (1) 6-HO-BOL (*m/z* 489 + 444 + 354); (2) 6-HO-ADD (*m/z* 458 + 413 + 323); (2') X-HO-ADD (*m/z* 458 + 413 + 323); (3) ADD (double peak) (*m/z* 370 + 341 + 325); (4) 17β-BOL (double peak) (*m/z* 401 + 372 + 356); (5) 17α-BOL (double peak) (*m/z* 401 + 372 + 356); and 6 + 7 + 8) 5ξ-AE (*m/z* 403 + 358 + 268).

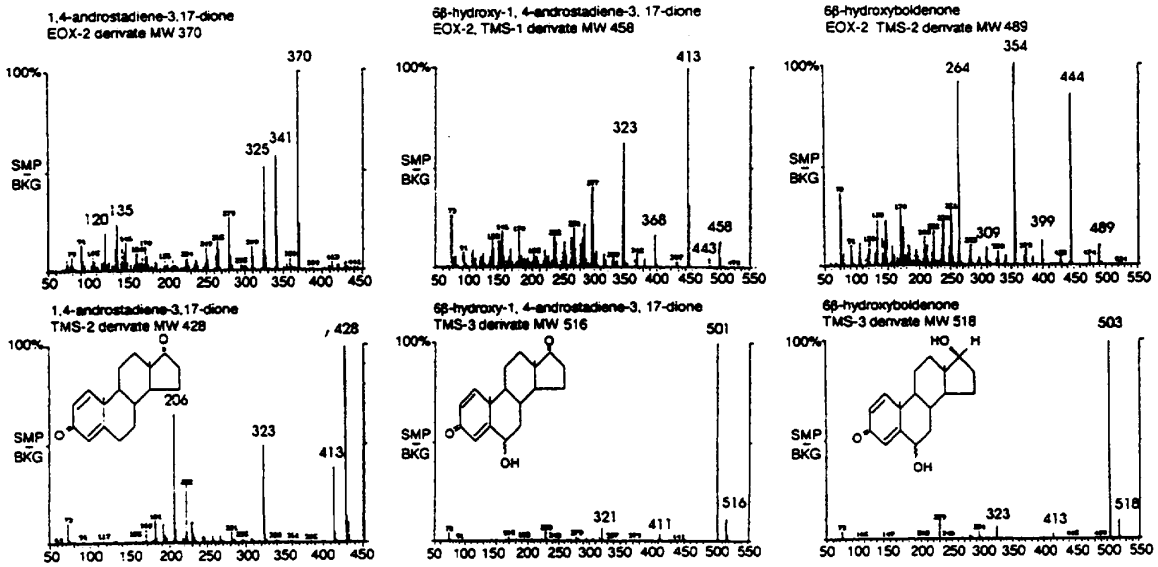

Fig. 2 Full scan electron impact (EI) mass spectra of 6-HO-BOL, 6-HO-ADD and ADD (EOX-TMS and TMS derivatives).

in vitro incubations (Table 1). 5β-AED was synthesized according to the method of Schänzer and Donike.[1].

In vivo results

Urine samples (R. Schilt) collected after administration of 17β-boldenone undecanoate were screened for all metabolites characterized during the *in vitro* experiments. 17β-BOL, 17α-BOL, ADD, 5β-AED and several reduction products of 17β-BOL and/or ADD were detected. The two prominent excretion products, were found to be 17α-BOL and an unidentified reduction product (5ξ-AEVI), eluting in HPLC fraction VI. In addition, a small amount of 6-HO-17β-boldenone was found. However, the detection of the 6-HO metabolites of ADD and 17β-BOL was hampered by strong matrix interferences. Therefore, the 6-HO-ADD and 6-HO-BOL HPLC fractions were fractionated again in smaller fractions. All fractions were analysed separately.

A similar metabolic profile was observed in the urine of the second animal (H. De Brabander). After administration, urine and feces samples were collected daily for 10 d. In urine 17α-BOL and two reduced metabolites, 5ξ-AEVI and 5ξ-AEVII, detected in HPLC fractions VI and VII, respectively, were the most prominent metabolites. ADD, 5β-AED and the HO metabolites were of minor quantitative importance. 6-HO-BOL and 6-HO-ADD were detected during the first few days only.

The quantitative and qualitative differences between the excretion in feces and urine are both striking and illustrated by the results summarized in Table 2 and 3. The hydroxy metabolites, 17β-BOL and ADD, were absent in feces. Instead, small amounts of 17α-BOL, 5β-AED and other reduced metabolites were found. In feces, the metabolite of HPLC fraction VII was detected for at least 4 d after the detection of 17α-BOL. 5β-AED was detected in feces for only 3 d and in urine for at least 10 d after administration.

Routine screening

Upon screening of routine feces samples found positive for 17α-BOL, important additional information was gained by the isolation of other metabolites. Fifty routine feces samples found positive for 17α-BOL and 19 feces samples found negative for boldenone were screened for the presence of ADD and 17β-BOL. ADD was found in all 50 17α-BOL samples but only 17 were found positive for 17β-BOL. None of the 17α-BOL negative samples were found positive for ADD nor 17β-BOL. The presence of ADD, 17β-BOL and the large amounts of 17α-BOL found are remarkable. Excreted levels as high as, *e.g.*, 240,

Table 1 Review of the boldenone metabolites identified in both *in vitro* and *in vivo* experiments

Compound	Sample[a]	MM[b] TMS	MM[b] EO-TMS	MU[b] TMS	MU[b] EO-TMS	HPLC fraction	Diagnostic ions TMS	Diagnostic ions EO-TMS	ID status[c]
17β-BOL	M/L/Au	430	401	26.48	27.12/27.22	IV	430, 415, 325, 206	401, 372, 356, 135, 120	1
ADD	M/L/Au	428	370	26.26	27.69/27.79	III	428, 413, 323, 206	370, 341, 325, 135, 120	1
6-HO-ADD	M/L/Au	516	453	27.61	28.44	I	516, 501, 411, 321	458, 443, 413, 323	1
6-HO-BOL	M/L/Au	518	489	27.74	28.18	I	518, 503, 413, 323	489, 444, 354, 264	2
17α-BOL	L/Au.f	430	401	26.08	26.59/26.68	V	430, 415, 325, 206	401, 372, 356, 135, 120	2
5ξ-AE VI	L/Au	432	403	25.20	25.60	VI	432, 417, 327, 290, 275	403, 388, 358, 343, 313	2
5ξ-AE VII	L/Au.f	432	403	23.83	25.44	VII	432, 417, 327, 290, 275	403, 388, 358, 313, 268	3
5ξ-AE	L/Au	432	403	—	25.87	VI	432, 417, 327, 290, 275	403, 388, 358, 313, 268	3
5ξ-AE	L/Au	432	403	—	26.34	VI	432, 417, 327, 290, 275	403, 388, 358, 313, 268	3
5ξ-AE	L/Au	432	403	—	26.62	VI	432, 417, 327, 290, 275	403, 388, 358, 313, 268	3
X-HO-ADD	L	516	453	—	28.27	I	518, 503, 413, 323	489, 444, 354, 264	3
5β-AED	Au.f	430	372	24.46	27.05	V	430, 415, 325,	372, 357, 327, 267	3

[a] M = microsomes; L = isolated hepatocytes; Ax = animal experiments (x = u for urine, x = f for feces, x = u.f for urine and feces). [b] MM = molecular mass; MU = methylene units; EO-TMS = ethoxime-trimethylsilyl derivative; TMS = trimethylsilyl derivative. [c] Identification status: 1 = comparison with commercial reference product; 2 = synthesised; 3 = tentative identification from the mass spectrum.

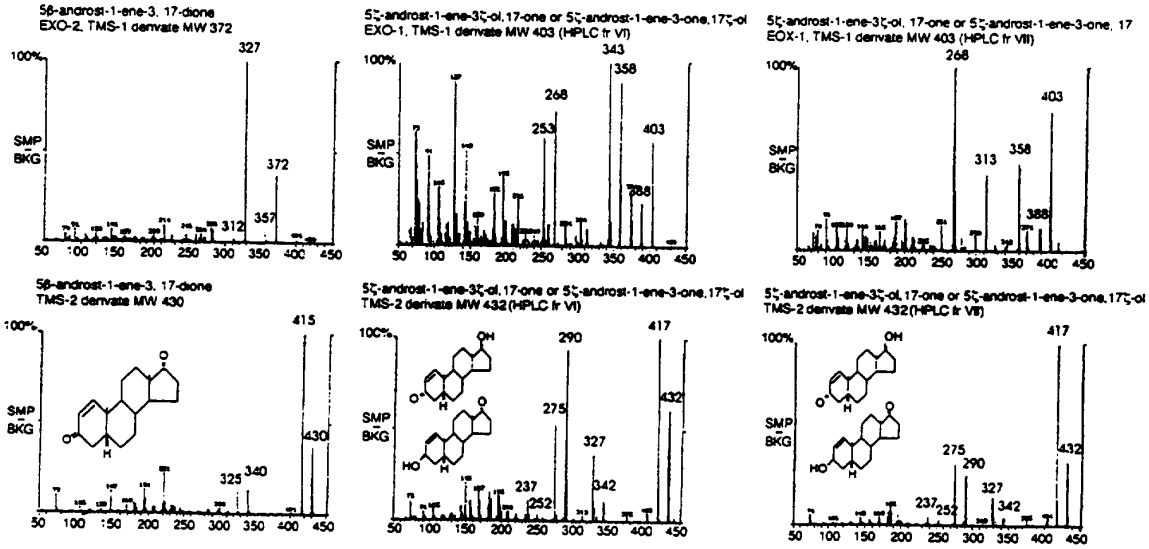

Fig. 3 Full scan EI mass spectra of the 5β-AED and two other metabolites (5ξ-AE) found in liver cell extracts after incubation with 17β-BOL and *in vivo* urine and feces samples (EOX-TMS and TMS derivatives).

24 and 632 ng g^{-1} were found for 17α-BOL, 17β-BOL and ADD, respectively. These findings, not corresponding with the amounts found after intramuscular administration of 17β-BOL, tend to suggest possible oral administration of boldenone or one of its esters. It is clear that further research is necessary on the dependence of the excretion profiles of boldenone metabolites on type of breed, method of application (*e.g.*, intramuscular, oral, transdermal), type of esters and interaction with co-administered drugs. Of 15 samples positive for 17α-BOL and ADD, fractions V–VII were analysed. In these fractions spectra of 5β-AED, 5ξ-AEVI and 5ξ-AEVII were found, which further substantiates the identification of boldenone in the field samples.

Moreover, large amounts of androst-4-ene-3,17-dione (AED) were found (Fig. 4). Levels as high as, *e.g.*, 196 ng g^{-1} were present in feces samples positive for 17α-BOL and ADD. In

Table 2 Urinary excretion levels of 17β-BOL and its metabolites after application of 700 mg 17β-boldenone

Time after application/d	17α-BOL/ ng ml^{-1} [a]	17β-BOL/ ng ml^{-1} [a]	ADD/ng ml^{-1} [a]	5β-AED/ ng ml^{-1} [b]	5ξ-AEVI ratio/IS[c]	5ξ-AEVII ratio/IS[c]
Blank	0	0	0	0	0	0
1	988	160	46	167	40.6	1.5
2	75	19	4	16	5.87	0.64
2[d]	200	31	5	28	10.01	0.27
3	356	37	5	46	4.08	0.27
4	38	9	4	13	3.6	0.14
5	139	15	1	18	2.75	0.18
7	30	18	2	9	2.09	0.18
8	14	5	0	20	0.48	0.07
9	15	4	0	3	0.48	0.07
10	5	0	0	5	0.09	0

[a] Results based on a five point calibration curve, by injection of direct control samples from the pure standards. [b] Results based on a five point calibration curve, by injection of direct control samples of 5α, AED. The EO, TMS derivative of 5β-AED has a similar mass spectrum to the EO-TMS derivative of 5α, AED. [c] Ratio of the sum of the abundances of the masses *m/z* 403, 358 and 268 of the EO-TMS derivatives of 5ξ-AEVI or 5ξ-AEVII to the sum of the masses m/z 407, 332 and 242 of the internal standard (IS) 5α-estrane-3β,17α-diol (5 ng μl). [d] Sample collected in the afternoon (all other samples collected in the morning) of the second day after application of boldenone.

Table 3 Fecal excretion levels of 17β-BOL and its metabolites after application of 700 mg 17β-boldenone

Time after application	17α-BOL/ ng g^{-1} [a]	17β-BOL/ ng g^{-1} [a]	ADD/ng g^{-1} [a]	5β-AED/ ng g^{-1} [b]	5ξ-AEVI ratio/IS[c]	5ξ-AEVII ratio/IS[c]
Blank	0	0	0	0	0	0
1	0	0	0	0	0	0.04
2	5	0	0	5	0	0.30
2[d]	7	0	0	10	0	0.18
3	3	0	0	3	0	0.14
4	4	0	0	0	0	0.16
5	4	0	0	0	0	0.13
7	4	0	0	0	0	0.15
8	5	0	0	0	0	0.03
10	0	0	0	0	0	0.03
11	0	0	0	0	0	0.07

[a] Results based on a five point calibration curve, constructed with the commercially available pure standards. [b] Results based on a five point calibration curve, by injection of direct control samples of 5α-AED. The EO-TMS derivative of 5β-AED has a similar mass spectrum to the EO-TMS derivative of 5α-AED. [c] Ratio of the sum of the abundances of the masses *m/z* 403, 358 and 268 of the EO-TMS derivatives of 5ξ-AEVI or 5ξ-AE to the sum of the masses *m/z* 407, 332 and 242 of the internal standard (IS) 5α-estrane-3β-7α-diol (5 ng μl^{-1}). [d] Sample collected in the afternoon (all other samples collected in the morning) of the second day after application of boldenone.

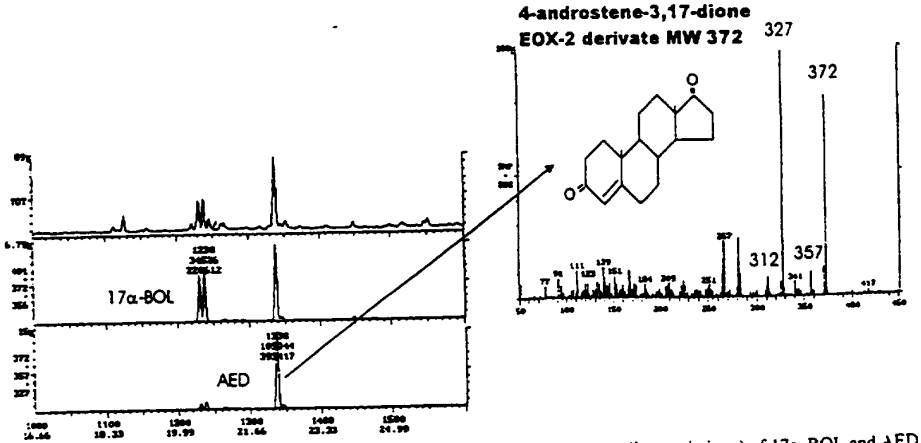

Fig. 4 Total ion current chromatogram and ion chromatograms (sum of the three most important diagnostic ions) of 17α-BOL and AED in a routine feces sample found positive for 17α-BOL (HPLC fraction IV, EOX-TMS derivatives).

only seven out of the 19 routine feces samples negative for 17α-BOL and ADD were traces of AED found. AED is present at various stages of the metabolism of steroids as an endogenous compound and as a conversion product of testosterone.[15] Physiological levels of AED were determined earlier in more than 2000 urine samples of cattle and veal calves and were found to be lower than 1 ng ml.[15] As for the presence of 17α-BOL and its metabolites, it is not clear what the origin of AED in these feces samples is. It cannot be excluded that its presence together with boldenone metabolites in the routine feces samples points to the possible co-administration (most likely oral) of naturally occurring anabolic steroids such as AED itself or testosterone with boldenone or one of its esters.

4. Conclusions

Several metabolites were identified after administration of 17β-BOL and after *in vitro* incubations with hepatocytes and subcellular fractions of bovine liver cells. We believe that some of these metabolites could be helpful in identifying the abuse of 17β-BOL for meat production in cattle.

17α-BOL has been described by Arts *et al.*[5] as endogenous in cattle urine. As a consequence, the identification of 17α-BOL in field samples does not demonstrate the illegal administration of 17β-BOL. According to Arts *et al.*,[5] the administration of 17β-BOL or one of its esters is compatible with the presence of 17β-BOL at levels above 1–2 ng ml^{-1} in urine.[5] This rule was only applicable for the urine samples in the *in vivo* experiments described here. With respect to detection in feces, our results demonstrate that 17α-BOL, 17β-BOL and/or ADD are less important. None of the feces samples met the criterion. Despite the number of 17β-BOL related metabolites present, they have to be declared negative. As a consequence, it would be better to consider the reduced forms of boldenone metabolites also.

We could identify 17β-BOL metabolites, found earlier after treatment with 17β-BOL, in 17α-BOL positive routine feces samples. The presence of these metabolites of boldenone supports the identification of 17α-BOL. This additional information is important because no substantial evidence was found in Belgium for the abuse of boldenone or one of its esters in cattle breeding. Because of the possible endogenous origin of 17α-BOL and, as a consequence, also of its Δ^1 unsaturated bond, and the lack of substantial evidence for the abuse of boldenone or one of its esters, these samples were considered 'negative'. It is still not clear whether these 'negatives' are true negatives since we could not detect any 17β-BOL metabolite in urine and feces samples of our 17β-BOL treated animals, before treatment. If 17α-BOL can be produced endogenously, the amounts produced, most likely, are small. We therefore propose to carry out further research on boldenone metabolites to set upper limits for boldenone related substances, other than 17α-BOL and 17β-BOL, such as 5ξ-AEVI and 5ξ-AEVII in urine and feces, providing most likely additional evidence for the illegal administration of 17β-BOL.

References

1 W. Schänzer and M. Donike, *Biol. Mass Spectrom.*, 1992, **21**, 3.
2 M. C. Dumasia and E. Houghton, *Biomed. Environ. Mass Spectrom.*, 1988, **17**, 383.
3 M. C. Dumasia and E. Houghton, *Biomed. Mass Spectrom.*, 1983, **10**, 434.
4 L. O. G. Weidolf, E. D. Lee and J. D. Henion, *Biomed. Environ. Mass Spectrom.*, 1988, **15**, 283.
5 C. J. M. Arts, R. Schilt, M. Scheurs and L. A. Van Ginkel, in *Proceedings of the Euroresidue III Conference*, Veldhoven, 6–8 May 1996, ed. N. Haagsma and A. Ruiter, University of Utrecht, Utrecht, The Netherlands, 1996, p. 212.
6 M. Van Puymbroeck, E. Royackers, R. F. Witkamp, L. Leyssens, A. S. Van Miert, J. Gelan, D. Vanderzande and J. Raus, in *Proceedings of the Euroresidue III Conference*, Veldhoven, 6–8 May 1996, ed. N. Haagsma and A. Ruiter, University of Utrecht, Utrecht, The Netherlands, 1996, p. 808.
7 P. O. Seglen, *Cell Biol.*, 1976, **13**, 29.
8 Van't Klooster, F. M. A. Woutersen-van Nijnanten, W. R. Klein, B. J. Blaauboer, J. Noordhoek and A. S. J. A. M. van Miert, *Xenobiotica*, 1992, **22**, 523.
9 L. Leyssens, E. Royackers, B. Gielen, M. Missotten, J. Schoofs, J. Czech, J. P. Noben, L. Hendriks and J. Raus, *J. Chromatogr.*, 1994, **654**, 43.
10 L. Hendriks, D. Courtheyn, B. Gielen, L. Leyssens and J. Raus, in *Proceedings of the Euroresidue II Conference*, Veldhoven, 3–5 May 1993, ed. N. Haagsma, A. Ruiter and P. B. Czedik-Eysenberg, University of Utrecht, Utrecht, The Netherlands, 1993, p. 367.
11 *Handbook of Derivatives for Chromatography*, ed. C. Blau and J. M. Halket, Wiley, Sussex, UK, 2nd edn., 1993.
12 R. R. Brown, R. Bain and V. C. Jordan, *J. Chromatogr.*, 1983, **272**, 351.
13 M. Niede and H. Jaeger, *J. Chromatogr.*, 1987, **413**, 207.
14 D. Courtheyn, J. Vercammen, H. De Brabander, I. Vandenreyt, P. Batjoens, K. Vanoosthuyze and C. Van Peteghem, *Analyst*, 1994, **119**, 2557.
15 G. C. Gatti, G. Zoboli, M. Zucchi, V. Ramazza and C. Cantoni, *Anal. Chim. Acta*, 1993, **275**, 105.

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