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Presence and metabolism of the anabolic steroid boldenone in various animal species: a review

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The review summarizes current knowledge on the possible illegal use of the anabolic steroid boldenone. The presence of boldenone and metabolites in different animal species and the possibility of the occurrence of endogenous boldenone and metabolites is assessed, as are the methods of analysis used for detection. Different laboratories in the European Union have examined the occurrence of boldenone and its metabolites. The results were discussed at different meetings of a European Commission DG-SANCO Working Party and summarized in an expert report. The situation of the different laboratories at this time is also covered herein. The overall conclusion of the Working Party was that there was a necessity for further research to distinguish between naturally occurring and illegally used boldenone forms. The confirmation of the presence of boldenone metabolites (free and conjugated forms) in certain matrices of animals is proposed as a marker for the illegal treatment with boldenone.

Keywords: boldenone, endogenous, illegal treatment, bovine, calves, anabolic steroids, phytosterol, metabolism

Introduction

For a number of years, boldenone has been increasingly detected in a number of biological samples in different European Union Member States. The question thus arose about whether this increased number of boldenone findings was due to the illegal treatment of animals or whether, in some circumstances, boldenone could be of endogenous origin. For instance, it was demonstrated that boldenone could be formed from phytosterols present in vegetable fat (Poelmans *et al.* 2003). In this respect, the substitution in animal feed of fat from animal origin (as beef tallow) by fat from vegetable origin, due to the crises from bovine spongiform encephalopathy and polychlorobiphenyl, might be important. Sometimes, phytosterol-enriched products are also recommended as animal feed. The phenomenon of increased boldenone detection might also be linked to the increased analytical capabilities, such as better limits of detection (LODs), of the European laboratories as a function of time. Several authors investigated this problem. Most experience was obtained with respect to the presence or 'absence' of boldenone and metabolites in urine from cattle and veal calves. Within the European Union, Belgium in contrast with other countries has much experience with the analysis of boldenone in bovine faeces samples.

At a meeting of the SANCO Working Party (25 February 2003) in Brussels with the European Union Commission, Community Reference

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Laboratory (CRL) and national experts, it was decided that an expert report on the current state-of-the-art of the metabolism of boldenone should be written as soon as possible. Laboratories from Belgium, France, Italy and the Netherlands volunteered to participate in the expert group and Belgium (Professor Dr Hubert De Brabander) coordinated the report. It was also decided that once the report was finalized, a condensed updated version had to be published in a peer-reviewed journal.

General information on boldenone

17β-Boldenone (17β-Bol), also called 1-dehydrotestosterone, androsta-1,4-diene-17β-ol-3-one, is a steroid with androgenic activity that differs from 17β-testosterone (17β-T) by only one double bond at the 1-position. Important steroids closely related to 17β-Bol and 17β-T are the 17β-boldenone epimer, i.e. 17α-boldenone, androsta-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (AED). These two diketo substances, ADD and AED, are precursors of 17β-Bol and 17β-T, respectively, in humans and different animal species. Their chemical structures are shown in figure 1.

17β-Bol, esters of 17β-Bol (e.g. undecylenate ester) and ADD are for open sale as anabolic preparations. 17β-Bol improves the growth and feed conversion of cattle and therefore might be abused to achieve more efficient meat production. Boldione (ADD) is sold on the Internet for use by bodybuilders as a product with an even greater anabolic potency than 17β-Bol itself. One remarkable feature is that—without a methyl group protecting the 17-OH group—17β-Bol is active after oral administration in humans. Other interesting molecules are 5β- and 5α-AED (figure 2) in which the double bond is in the 1-position instead of in the 4-position. Not all official laboratories performing routine veterinary drug residue testing can measure all these 'new' substances and some are even unaware of their existence.

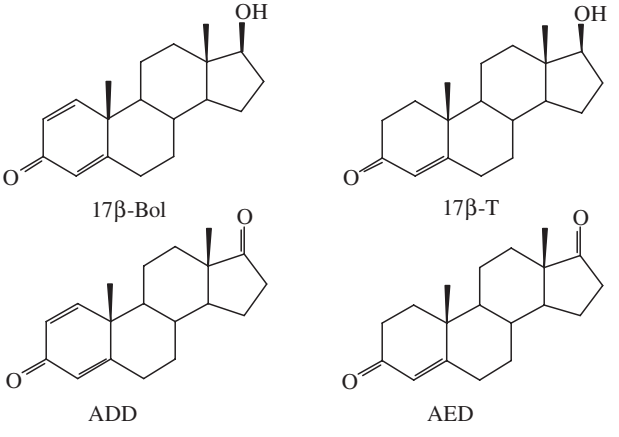

Figure 1. Structures of 17β-boldenone (17β-Bol) and 17β-testosterone (17β-T) (above) and of androsta-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (AED).

The metabolism of 17β-T, 17β-Bol and other related substances is under investigation with animal experiments by laboratories of the expert group. Like the other androgenic steroids, 17β-Bol is classified by the International Agency for Research on Cancer (IARC) as a probable human carcinogen, with a carcinogenicity index higher than that of other androgens, such as nandrolone, stanozolol, testosterone and clostebol. A recent study has also demonstrated the role of 17α-Bol in the development of human prostate carcinomas implanted in mice (Baisch *et al.* 1998).

Analytical methods dedicated to boldenone

17β-Bol and metabolites can be analysed by both gas and liquid chromatography coupled to multiple mass spectrometry (GC-MS^n and LC-MS^n). The academic literature discusses both analysis methods widely. Schänzer and Donike (1993) and Van Puymbroeck *et al.* (1998) described the use of GC-MS^n methods for the detection of trimethylsilyl (TMS) derivates, analysed in electron impact (EI) mode. Van Poucke and Van Peteghem (2002) and Nielen *et al.* (2004) published results of the detection

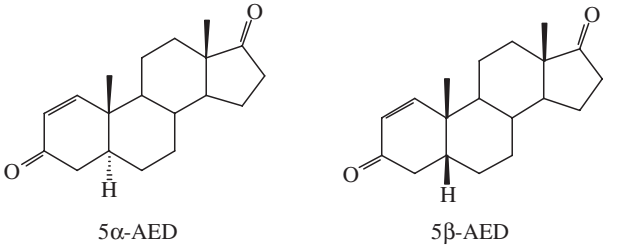

Figure 2. Chemical structures of the molecules 5α-androst-1-ene-3,17-dione (5α-AED) and 5β- androst-1-ene-3,17-dione (5β-AED).

with LC-electrospray ionization (ESI)-MS in positive-ion mode, and Draisci *et al.* (2003) discussed the results with an atmospheric pressure chemical ionization (APCI) interface in positive-ion mode.

In 2002, the CRL analysed the results of an inquiry into 17β-Bol analyses in the European Union Member States (Jonker *et al.* 2002). It was concluded that the situation between the Member States was distinctly different, e.g. operating with different LODs, demonstrating a need to be harmonized. Harmonization requires that each Member State can detect both 17α- and 17β-Bol in their national control programme with a validated method according to Commission Decision 2002/657/EC (2002) at the CRL-National Reference Laboratory (NRL) proposed minimum required performance level (MRPL) for both 17α- and 17β-Bol in urine of 1 $\mu g\,kg^{-1}$ (ppb).

Investigation of boldenone metabolism in various species

After the intramuscular administration of radioactive-labelled 17β-Bol to castrated male horses, the formation of metabolites was investigated by excretion of radioactive metabolites in urine (Dumasia *et al.* 1983).

Studies on human volunteers showed that after administration of 17β-Bol, it was excreted as a conjugate in urine (Schänzer and Donike 1993). Galletti and Gardi (1971) and Cartoni *et al.* (1998) described the formation and excretion of metabolites of 17β-Bol and 17β-Bol itself in urine after an oral administration of 17β-Bol to human volunteers.

Although 17β-Bol was only rarely detected in injection sites and/or preparations (Vanoosthuyze *et al.* 1994), questions arose about its use. *In vitro* and *in vivo* metabolism of 17β-Bol was investigated by Van Puymbroeck *et al.* (1998b). Two different types of liver preparations (microsomes and monolayer cultures of isolated hepatocytes) were used to identify important metabolites *in vitro*. The main metabolite produced by microsomes was ADD, while in the isolated hepatocytes, 6-OH-17β-Bol and 6-OH-ADD were identified.

This research group also examined the excretion of 17β-Bol in a calf and a cow. The main metabolite found in urine was 17α-Bol. Some reduced, oxidized (such as ADD) and hydroxylated metabolites were also found. Faeces samples were investigated for the presence of 17α-Bol and 5β-AED, which does not naturally occur in bovines, and other reduced metabolites. No 17β-Bol was detected in bovine faeces. Faeces samples showed a different metabolite profile in comparison with urine samples. No hydroxylated or oxidized products were found (Van Puymbroeck *et al.* 1998a). In the urine of a male calf treated intramuscularly with 200 mg 17β-Bol-undecylenate, 17α- and 17β-Bol, and also ADD and 5β-AED were identified. In a second experiment, a mature cow was treated intramuscularly with 700 mg free, unesterified 17β-Bol. The metabolic profile in urine was comparable. Besides 17α- and 17β-Bol, 5β-AED was found at levels comparable with 17β-Bol, while ADD was found at lower concentrations. The differences in excretion in faeces were considerable. 17α-Bol and 5β-AED were the most predominant metabolites, whereas 17β-Bol and ADD were not detected (Van Puymbroeck 2000).

The presence of metabolites is most frequently investigated in the matrix urine. For a short overview of metabolites found in treated and untreated animals, see table 1.

Table 1. Metabolites of boldenone present in urine in different untreated and treated animal species.

	Untreated		Treated	
	Male	Female	Male	Female
Pig	17β-Bol	–	n.k.	n.k.
Cattle	17α-Bol	–	17α-Bol, 17β-Bol and metabolites	17α-Bol, 17β-Bol and metabolites
Horse	17β-Bol	n.k.	n.k.	n.k.

–, No metabolites present.
n.k., Not known.

Naturally occurring boldenone metabolite

Bol (α or β) has been detected in untreated animals of several animal species. The species and the gender are known. The presence of endogenous Bol (α or β) is mostly observed in male animals, but the kind of feeding is mostly unknown. The presence of 17β-Bol in the entire male pig has been demonstrated on several occasions. Ten extracts of boar testicles analysed by both gas chromatography-high resolution mass spectrometry (GC-HRMS) and LC-MS^n unequivocally contained 17β-Bol. The concentration of 17β-Bol varied between 1 and 20 $\mu g\,kg^{-1}$. The epimer 17α-Bol was not found ($< 0.1\,\mu g\,kg^{-1}$) because pigs do not have the enzyme to convert 17β-steroids into 17α-steroids. Further ADD, 17β-nortestosterone (17β-NT), norandrostendione (NAED a precursor of NT), 17β-oestradiol (E2), 17β-T and AED were detected.

In a joint project of the Laboratory of Chemical Analysis (LCA), the European Union Community Reference Laboratory for Residues (RIVM) and the Ecole Nationale Vétérinaire de Nantes (LABERCA) in cooperation with the US Department of Agriculture (USDA) (PIGSTER Project: Dr Michael Hoffman), a large number of samples (urine, testis, kidney, liver and meat) from boars, cryptorchids, barrows, gilts and sows were analysed. The main target compound was 17β-NT, but a large number of analytes were measured, including 17β-Bol, ADD, 17β-T and AED. It was demonstrated in all three laboratories that 17β-Bol was present in the testis and urine of entire male pigs, including cryptorchids (De Wasch *et al.* 2003).

In addition, it was observed that 17β-Bol was not present in samples of female porcine animals. However, note that samples of an intersex were recently analysed. Since this (type of female) animal has one testis, male hormones were also found (Van Cruchten *et al.* 2002).

Since 17β-Bol can also be abused for horse doping, the possible endogenous presence of 17β-Bol is also a subject of concern for horseracing doping laboratories. It was demonstrated that 17β-Bol is present in entire male horses (E. N. M. Ho, unpublished data, 2002).

Arts *et al.* (1996) reported the presence of 17α-Bol in concentrations varying from less than 0.1 to 2.7 $\mu g\,kg^{-1}$ in urine samples of untreated calves. In the same samples, only traces of 17β-Bol were found (0.01–0.1 $\mu g\,kg^{-1}$). In regulatory controls, concentrations in the range 0.2–0.7 $\mu g\,kg^{-1}$ were found. From those data, it can be concluded that 17α-Bol can be of natural origin in calves and cattle even when the animal has not been treated. The origin of the substance is not known.

In a routine screening programme described by Van Puymbroek (2000), 50 samples of faeces positive for 17α-Bol were analysed. ADD was predominant in all 50 samples, while 17β-Bol only occurred in 17 samples. Further research is necessary depending on the excretion profiles of 17β-Bol metabolites. In the samples in which 17α-Bol was found, high levels of AED were also seen. AED is a conversion product of testosterone and was examined as a marker for the use of testosterone (Gatti *et al.* 1993). The exact origin of AED in the faeces samples and the relationship with 17β-Bol is not known.

Possible origins of natural boldenone

Microorganism hypothesis

Microorganisms can selectively dehydrogenate steroids (Smith *et al.* 1989b). Barthakur *et al.* (1996) described the transformation of β-sitosterol (a plant steroid or phytosterol naturally occurring in plant material) into ADD in the presence of a 1(2)-dehydrogenase by the *Mycobacterium* species NRRL B-3683. As shown in figure 3, oxidation at positions 3 and 17 yields the steroid nucleus of (5β-)AED and/or ADD. A large number of naturally occurring phytosteroids are described by Shimada *et al.* (2001).

It has been demonstrated that precursors of 17β-Bol can be detected in the faeces of rats fed with phytosterols (Song *et al.* 2000). Galletti and Gardi (1971) revealed that humans could reduce ADD to 17β-Bol after oral intake. Furthermore, reversion of the reactions can occur under aerobic conditions.

The microbial enzymatic reduction of ADD to AED, 17β-T and 17β-Bol was described by Goren *et al.* (1983). Two reducing activities observed in washed cell suspensions and cell-free extracts of *Mycobacterium* spp. NRRL B-3805 were found to account for these bioconversions. One was a

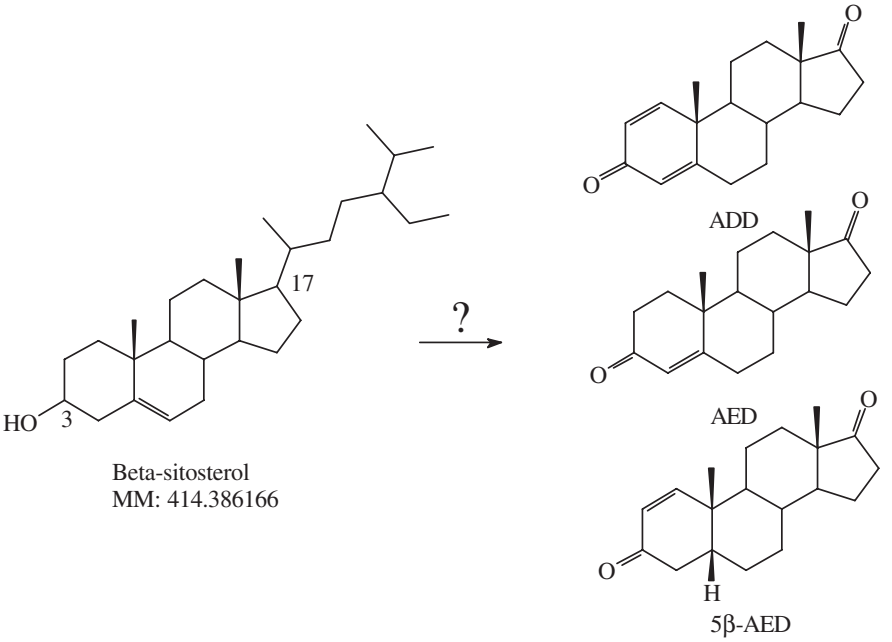

Figure 3. Possible transformation of β-sitosterol into AED and/or ADD.

1-ene-steroid reductase, the other a 17-keto steroid reductase. *Mycobacterium* spp. VKM Ac-1815D strain could cleave the sterol side chain resulting in AED as a major product with a molar yield of 63–68% (Egorova *et al*. 2002). Mutants were obtained that retained the ability to produce AED from sitosterol with molar yields of 70–75%. This approach offered the possibility of obtaining improved biocatalysts for AED or 17β-T production from sterols. *Phycomyces blakesleeanus* (filamentous fungi) transformed progesterone (P), 17β-T and AED into mixtures of products (Smith *et al*. 1989a). Jenkins *et al*. (2001) identified AED in a river containing paper mill effluent. Effluent from a paper mill discharging into the Fenholloway River, Taylor County, Fl, USA, contained chemicals that masculinize females of the resident population of eastern mosquito fish (*Gambusia holbrooki*). Induced androgen receptor-dependent transcriptional activity was observed in transient transfection cell culture assays. The presence of AED was confirmed by LC-MS. In a second study, Jenkins *et al*. (2003) identified and characterized steroids in the Fenholloway sediment. AED and P were confirmed in the river sediment at concentrations greater than in the river water column. The data were consistent with the hypothesis that pine pulp-derived phytosteroids in the paper mill effluent accumulate in river sediment, where microbes convert them into P and then into AED and other bioactive steroids.

Studies in invertebrates

The invertebrate *Neomysis integer* (Crustacea; Mysidacea) was used as an alternative model for the partial replacement of vertebrate animals in metabolism studies with illegal growth promoters and veterinary drugs (De Wasch *et al*. 2002, Verslycke *et al*. 2002). Vertebrate-type steroids such as 17β-T have been used as substrates to study enzyme systems (cytP450) of the oxidative metabolism in invertebrates. Results from these studies provide information on the degree of similarity to the enzyme systems in vertebrates.

N. integer was exposed to 17β-T in a study to assess testosterone metabolism in order to provide information on its metabolism capacity and thus susceptibility to contaminants in the environment. The extracts were tested for hydroxy metabolites, dihydro-T, 17β-Bol and AED. Extracts of *N. integer* after exposure to stanozolol, which is an exogenous anabolic steroid, were also tested for the same analytes. Surprisingly, small concentrations of 17β-Bol were

observed in both extracts. An explanation could be given for the formation of 17β-Bol after exposure to 17β-T, but it is more difficult to explain how 17β-Bol could be formed after administration of stanozolol. Somehow, the hormone metabolism is affected after administration of an endogenous or exogenous anabolic steroid. Only after exposure to a high concentration of an anabolic steroid (17β-T and also stanozolol), 17β-Bol was formed and detected in the medium (highest response) and organism (lowest response). Further studies with *N. integer* showed that phytosterols such as β-sitosterol can be transformed into 17β-T by the organism (Poelmans *et al.* 2003). *N. integer* produced AED from 17β-T. When AED was added to the organism, a mixture of 17β-T and ADD was formed. Finally, the addition of a solution of ADD to *N. integer* resulted in the formation of 17β-Bol. All the steps in the transformation of β-sitosterol into 17β-Bol have thus been investigated.

Further results and experiments from different Member States are now given.

The Netherlands

SKV and CBD. Tests were carried out for 17β- and 17α-Bol, natural hormones and the important metabolites ADD and AED in untreated animals. Two independent systems (called self-monitoring systems in the Netherlands) have been in operation for 12 and 8 years, respectively. For veal calves, this self-monitoring is carried out by the SKV (Stichting Kwaliteitsgarantie Vleeskalversector, or the Foundation for the Quality Guarantee of the Dutch Veal Calf Sector) and for cattle by the CBD (Controlebureau Dierlijke Sector, or the Control Office for the Animal Sector).

In the SKV (calves) programme, 17β-Bol but not 17α-Bol was included in the GC-MS screening in 1996. The presence of 17α-Bol was regarded as endogenous because of the presence identified in untreated animals (Arts *et al.* 1996). Somewhat later, 17β-Bol was added to the screening programme for cattle.

The use of GC-MS permitted the analysis of pooled urine samples, with a maximum of five samples from one farm per pooled sample. Where the presence of 17β-Bol was suspected, each sample taken at that specific farm was analysed as an individual sample using a confirmatory method. The same set up was used for the analysis of cattle urine samples taken by the CBD.

The relation between 17β-Bol and 17α-Bol in samples was investigated and compared with other studies. In line with the results of Van Puymbroeck *et al.* (1998b), the amounts of 17α-Bol were much higher than those of 17β-Bol. Furthermore, the measured levels were in many cases higher than the amounts found in guaranteed untreated animals.

Where 17β-Bol was found in a pooled sample, 17 corresponding samples of the same farm were tested for the presence of 17β-Bol, 17α-Bol, ADD and AED. In two samples, the presence of 17β-Bol was confirmed. In six other samples, the presence of 17β-Bol was suspected, but the identification criteria were not fulfilled (due to technical problems). ADD was detected in all samples. Furthermore, all samples, including the control samples, indicated the presence of AED. Androstanediols were not detected although this may be due to the LOD being too high in the full-scan measurements.

In recently analysed samples, the presence of ADD was indicated as a possible marker for the use of 17β-Bol. Neither the identity of ADD nor 17β-Bol was confirmed, but only a few ion fragments were analysed.

A similar comparison of 17β-Bol and 17β-T detected in pooled urine samples from mature cattle was performed. The number of occurrences of 17β-Bol and 17β-T were lower compared with the data for the veal calves. In general, 17α-Bol and 17β-Bol were found less frequently than in urine from veal calves.

All the screening results for 17β-Bol and 17α-Bol obtained from the analysis of pooled urine samples from calves (SKV) sampled between May 2000 and February 2002 were analysed by gender, age and date of sampling.

A total of 1329 pooled samples were analysed over this period. Of these samples, 36 (2.7%) showed an estimated level of $\geqslant 0.2\,\mu g\,kg^{-1}$ for 17β-Bol and 88 samples (6.6%) had an estimated level of $\geqslant 0.2\,\mu g\,kg^{-1}$ for 17α-Bol. The majority of the animals (>99%) sampled were males. It was impossible to trace the gender of all samples (>5000, i.e. 1329 pooled samples of three to five individual samples each). However, a selection of the samples with 17α-Bol levels $\geqslant 1.0\,\mu g\,kg^{-1}$ and/or 17β-Bol levels $\geqslant 0.2\,\mu g\,kg^{-1}$ was further investigated to obtain the gender and the age of the animals.

There was an indication that there was an effect of the sampling period because in certain periods (spring and autumn) the occurrence of 17α- and/or 17β-Bol was lower compared with other periods (summer and winter). Studies of the effect of the age of the animals proved that the age has no apparent influence on the presence of either boldenone isomer. Besides urine and faeces, other samples like the testicles of veal calves were also studied for the presence of steroids.

The SKV sampled testicles of five rosé (calves fed with complete diet) and six white meat calves (fed with milk replacer) at the slaughterhouse. The animals were part of the normal production cycle and not necessarily untreated animals. Neither 17β-Bol, 17α-Bol nor ADD was not detected in any of the samples. From this observation, it could be concluded that 17β-Bol and/or ADD were not endogenously produced in the testicles. There were no indications of the presence of 17β-NT, 17α-NT or NAED. As expected, 17β-T was found at relative high levels (50–350 $\mu g\,kg^{-1}$), together with 17α-T at lower levels (0.5–2.5 $\mu g\,kg^{-1}$) and AED (3–14 $\mu g\,kg^{-1}$). Finally, both epimers of E2 (17α- and 17β-) were present at the sub-$\mu g\,kg^{-1}$ level.

RIKILT and RIVM. In a study performed by RIKILT (Rijkskwaliteitsinstituut voor Land- en Tuinbouwproducten, or the Institute of Food Safety) and RIVM (Rijksinstituut voor Volksgezondheid en Milieu, or the National Institute for Public Health and the Environment), an experiment was carried out recently without any intentional relationship to the boldenone discussion. The objective of this governmental study by RIKILT was to obtain reference sample materials of untreated calves (Sterk *et al*. 1998).

Twenty-five male and 25 female calves were purchased from many different farms at the age of 2 weeks and kept at a farm under controlled experimental conditions. Access was restricted to authorized personnel only. The calves were fed with regular milk replacer. Veterinary medicines were administered only to treat individual diseases and infections, and each use was recorded. No other intentional administration of veterinary drugs took place. Based on this information, the sample materials from these calves have to be considered as blank materials at the time of sampling. Samples of urine and rectal faeces obtained from these animals (aged approximately 27 weeks) at the farm were analysed for the presence of 17α-Bol and 17β-Bol by both RIKILT and RIVM. None of these samples contained any of the two analytes at levels exceeding 0.1 $\mu g\,kg^{-1}$. However, in addition to the samples taken directly from the colon, samples were taken at slaughter (aged approximately 28 weeks) from the dried faeces stuck on the fur and skin of the animals. The presence of both 17α-Bol (12/20) and 17β-Bol (6/20) was detected and fully confirmed in some of these samples. Indicative concentrations ranged from about 1 to $>10\,\mu g\,kg^{-1}$ for 17α-Bol and from 0.1 to 2 $\mu g\,kg^{-1}$ for 17α-Bol. The samples were analysed with both LC-MS^n (Nielen *et al*. 2004) and GC-MS. These findings alerted the CRL and the Commission about the potential problems with the interpretation of findings of 17β-Bol in bovines.

Further studies into the quantitative aspects of the formation of 17α-Bol, 17α-Bol and ADD are currently ongoing. These studies include the effect of contamination of urine with (dried) faeces and the influence of this on the presence of boldenone-related compounds.

Italy

Since a positive case for boldenone residues in 1998, positive results in Italy have progressively increased from October 2000. The National Laboratories (Istituti Zooprofilattici Sperimentali) responsible for the monitoring results in the National Residue Plans (NRP) have found numerous cases of both 17α- and 17α-Bol. 17β-Bol has been found in bovine urine at levels even $>2\,\mu g\,kg^{-1}$ in some cases, and seems always to be associated with the presence of 17α-Bol. Of the samples found positive for 17β-Bol, four were in 2000, 38 in 2001 and 54 to May 2002. 17β-Bol concentrations in positive samples ranged from the LOD to 0.5 $\mu g\,kg^{-1}$ in 2000, from the LOD to 7 $\mu g\,kg^{-1}$ in 2001, and from 0.1 to 2 $\mu g\,kg^{-1}$ in 2002. 17α-Bol was detected at levels of 20 $\mu g\,kg^{-1}$ (2000), $>80\,\mu g\,kg^{-1}$ (2001) and 45 $\mu g\,kg^{-1}$ (2002).

Tests on more than 1500 samples of cattle urine (provided by Servizi Veterinari) showed that where 17β-Bol was found in one or more samples from a group of animals, 17α-Bol was constantly found in around 20% of samples from that group, thus giving a potential threshold for identification of illegal treatment even where 17α-Bol alone is found in a group of animals. Although monitoring is performed across Italy, positive samples have been found in only a limited number of farms in Lombardy, Piedmont,

Friuli Venezia Giulia and Emilia-Romagna. Following a positive finding by a routine on field laboratory (RFL), Italian official control measures require the analysis to be repeated by the NRL (Istituto Superiore di Sanità, or ISS) at the request of the producer. The same validated LC-MS^2 method is always used for NRL official control and research, thus allowing a persistent view of the situation. In recent years, the NRL has found 17α-Bol in more than 100 cattle, even at levels $>50\,\mu g\,kg^{-1}$ (average $11\,\mu g\,kg^{-1}$). More than 60% of farms investigated have been found positive for 17β-Bol, with maximum levels $>1.5\,\mu g\,kg^{-1}$ (average $0.5\,\mu g\,kg^{-1}$). In addition, more than 60% of these cattle have also been found positive for ADD, with levels even $>30\,\mu g\,kg^{-1}$ (average $3\,\mu g\,kg^{-1}$).

To verify the analytical facilities of the RFLs for the detection of boldenone and other steroids, the NRL performed an inventory of the analytical methods adopted by the National Laboratories and organized a ring test on boldenone and other steroids. The participant laboratories that communicated their results demonstrated an LOD of 0.04–$1\,\mu g\,kg^{-1}$ for 17α-Bol and of 0.03–$1\,\mu g\,kg^{-1}$ for 17β-Bol. This performance complies with the MRPL as proposed by the CRL of $1\,\mu g\,kg^{-1}$ for both 17α-Bol and 17β-Bol in bovine urine. Recently, the Carabinieri—Nucleo Antisofisticazioni e Sanità (NAS Carabinieri) discovered preparations for on-farm use—probably for oral treatments—containing a mixture of 17β-Bol, ADD and a 17β-Bol ester.

An *in vivo* study is in progress at the ISS–NRL on animals of the same breed, age and sex, as those found positive for 17β-Bol under the Official Control Programme. Animals are kept under the strict control of ISS staff. Analyses are carried out by a newly developed specific and sensitive LC-MS^2 (Draisci *et al.* 2003). Oral treatment of veal calves with 17β-Bol, 17β-Bol esters and/or ADD resulted in 17α-Bol (the main metabolite), 17β-Bol and ADD in all urine samples and demonstrated for the first time that ADD is a precursor of 17α-Bol and 17β-Bol in cattle. Following a suspension of treatment, 17β-Bol disappeared shortly afterwards. However, 17α-Bol residues remained for at least 25 days (observation period), with an average concentration of about $2\,\mu g\,kg^{-1}$. This is almost the same value reported in literature data for untreated animals (Arts *et al.* 1996).

Administration of just ADD led to levels of 17β-Bol higher than ADD in urine. However, administration of 17β-Bol esters or 17β-Bol and ADD gave similar or higher levels of ADD than 17β-Bol. The latter residue profile is that most often encountered in positive official control urine samples. ADD, 17α-Bol and 17β-Bol were not detected in the ISS experimental study in fresh faeces from untreated animals, thus confirming the data of previous studies (Van Puymbroeck *et al.* 1998a, b). On the other hand, 17α-Bol, ADD and 17β-Bol residues were found in fresh faeces from animals orally treated with ADD and 17β-Bol, with residue profiles similar to those found in urinary excretion profiles for the same treated animals (i.e. 17α-Bol > ADD $\geqslant$ 17β-Bol), although residue levels in faeces are usually higher than those in urine from the same treated animal. As expected, ISS preliminary data showed that the conjugates are present in the urine, while the free steroids are the main form in the faeces.

France

The Laboratoire d'Etudes des Résidus et des Contaminants dans les Aliments (LABERCA), the French National Reference Laboratory for growth promoter agents in food-producing animals, revealed since 2002 an increased number of 'positive cases' of boldenone in calf urine. In spite of feed and injection sites analysis, no reasonable and unambiguous explanation was available to explain excretion of 17α-Bol in all tested animals. At the end of 2002, LABERCA launched studies to determine steroid profiles after oral and intramuscular administration of 17β-Bol, ADD and 17β-Bol esters in bovine in collaboration with the ISS (Italy) and the SKV/CBD laboratories (TNO, the Netherlands). Nine metabolites were identified; only four were present whatever the treatment and the applied boldenone source. Amongst the most abundant metabolites, three were particularly interesting: 17α-Bol itself and metabolites 5β-androst-1-en-17α-ol-3-one and 5β-androst-1-en-17β-ol-3-one. These two metabolites were explored in naturally occurring boldenone urine; the first results (to be confirmed) tended to demonstrate that those metabolites are not present at low residue level and could be good candidate markers to discriminate in-between natural and misuse situations of boldenone and precursors in bovine. 17α-Bol remains the best metabolite to screen for possible use of boldenone or precursors in bovine whatever the administration route. Table 2 summarizes the situation and

Table 2. Natural occurring situation of boldenone and the strategy for the control of the use of boldenone in cattle.

	Normal situation	Strategy for boldenone control
Urine	Traces of 17α-Bol	17α-Bol conjugate $>2\,ng\,ml^{-1}$ SUSPICION of illegal use
	No 17β-Bol	Presence of 17β-Bol conjugate at any level CONFIRMATION of illegal use
Faeces	Free unconjugated 17α-Bol Free unconjugated 17β-Bol (in dried faeces)	?

the strategy to adopt in case of a suspected boldenone sample.

A Phase II metabolism study is ongoing with the same objectives than those exposed before. Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) measurement of 17α-Bol has been carried out after an injection of ADD and 17β-Bol; the potential of this technique is currently evaluated for urine and faeces samples characterized by high concentrations of 17α-Bol.

Conclusions

Based on the data described above, there cannot be any doubt that 17β-Bol and metabolites are naturally produced steroids in a variety of non-boldenone-treated species. A remarkable fact is that boldenone in non-treated animals is observed so rarely. No clear correlation has yet been found between the different observations. The presence of 17α- and 17β-Bol in dried faeces, present on the animals fur, from untreated male and female veal calves has been demonstrated. Recent research has shown that the presence of 17β-Bol conjugates at any level in urine from veal calves is proof of illegal treatment (17β-Bol conjugates are water-soluble forms of 17β-Bol bound to, for example, glucuronic acid formed through metabolism pathways after administration). Urine should thus be sampled without faecal contamination to avoid misinterpretation. For regulatory purposes, 17β-Bol residue findings must always be specified as free or conjugated 17β- and/or 17α-Bol with the explicit identification of the animal species. For retrospective evaluation of the data, it is useful also to specify the breed, gender and age of the animals.

The formation of 17α- and 17β-Bol from phytosterols has been demonstrated in *in vitro* conditions and in using an invertebrate model. Limited studies on the formation of Bol metabolites from phytosterols *in vivo* in bovines are ongoing. Further research is necessary.

The aim was to find ways of distinguishing between the presence of naturally occurring boldenone forms and the presence of these substances due to their illegal administration. Criteria must be developed as soon as possible to allow the competent authorities to take appropriate action. The discrimination between the conjugated and the free form of 17β-Bol in urine is a first indication to distinguish an endogenous presence or illegal use. Further research is suggested concerning metabolism studies with veal calves in order to look for a marker residue indicating administration of 17β-Bol and including phase II metabolites as potential markers of an illegal use of Bol or one of its precursors. Studies on the formation of 17β-Bol in faeces (including dried faeces present on the animals fur), the possible transfer of the substance to urine and metabolization studies with alternative models, like the invertebrate *Neomysis integer*, are recommended.

Appendix: Abbreviations

17α-Bol	17α-boldenone	1,4-androstadiene-17α-ol-3-one
17α-NT	17α-nortestosterone	17α-hydroxy-19-norandrost-4-en-3-one
17α-T	17α-testosterone	4-androstene-17α-ol-3-one
17β-Bol	17β-boldenone	1,4-androstadiene-17β-ol-3-one

17β-NT	17β-nortestosterone	17β-hydroxy-19-norandrost-4-en-3-one
17β-T	17β-testosterone	4-androstene-17β-ol-3-one
5α-AED	5α-androstenedione	5α-androst-4-ene-3,17-dione
5β-AED	5β-androstenedione	5β-androst-4-ene-3,17-dione
ADD	androstadienedione	1,4-androstadiene-3,17-dione
AED	androstenedione	androst-4-ene-3,17-dione
dihydro-T	dihydrotestosterone	17β-hydroxy-5α-androstan-3-one
E2	oestradiol	3,17β-dihydroxy-1,3,5(10)-estratriene
NAED	norandrostenedione	19-nor-4-androstene-3,17-dione
P	progesterone	4-pregnene-3,20-dione

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