Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 626–632

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Estranediols profiling in calves' urine after 17β-nandrolone laureate ester administration☆

Gaud Pinel[a,*], Lauriane Rambaud[a], Fabrice Monteau[a], Christopher Elliot[b], Bruno Le Bizec[a]

[a] Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA[1]), École Nationale Vétérinaire de Nantes, Route de Gachet, BP 50707, 44307 Nantes cedex 3, France
[b] Agri-food and Land Use, Queen's University, Stranmillis Road, Belfast BT9 5AG, Northern Ireland, United Kingdom

ARTICLE INFO

Article history:
Received 2 October 2009
Received in revised form 4 December 2009
Accepted 25 January 2010

Keywords:
17β-Nandrolone
Estranediols
Metabolism
Calves
Urine

ABSTRACT

17β-Nandrolone (17β-NT) is one of the most recurrent forbidden anabolic steroid used in meat producing animals breeding. Because efficient control must both take into account metabolic patterns and associated kinetics of elimination, the metabolism of 17β-NT in bovines has already been investigated and is well documented, but only focussing on its main metabolites (i.e. 17α-nandrolone, 19-noretiocholanolone and 19-norandrostenedione). The goal of the present study was to enlarge this panel of 17β-NT metabolites, especially through the urinary estranediols fraction in order to perform a more global steroid profiling upon 17β-nortestosterone laureate ester administration in calves. A GC–MS/MS method has been developed to monitor and quantify 5 estranediols isomers including 5α-estrane-3β,17β-diol (*abb*), 5β-estrane-3α,17β-diol (*bab*), 5α-estrane-3β,17α-diol (*aba*), 5α-estrane-3α,17β-diol (*aab*) and 5β-estrane-3α,17α-diol (*baa*). Their urinary elimination kinetics have been established allowing detection of 4 estranediols up to several days after administration. All animals demonstrated homogeneous patterns of elimination both from a qualitative (metabolite profile) and quantitative point of view (elimination kinetics in urine). 5α-Estrane-3β,17α-diol (*aba*) was found as the major metabolite with concentrations up to 100 μg L^{-1}.

1. Introduction

The use of anabolic compounds is prohibited in food producing animals in the European Union [1]. Amongst other steroids, 17β-nandrolone (17β-19-nortestosterone) (17β-NT) may be used for its anabolic activity comparable to that of testosterone with less androgenic associated side effects. Detection of anabolic steroid abuse is performed through effective monitoring, detection, identification and confirmation methods. In this context, gas chromatography coupled to mass spectrometry (GC–MS) is the technique commonly used for the analysis of urine samples by anti-doping or analytical laboratories [2–5]. Improved analytical sensitivity, leading to increased periods of detection post drug administration, can be achieved in urine using gas chromatography–tandem mass spectrometry (GC–MS/MS) [6–9] or liquid chromatography–tandem mass spectrometry (LC–MS/MS) [10–15].

Efficient control of the illegal use of anabolic steroids must both take into account metabolic patterns and associated kinetics of elimination [5,8,16] which have mainly been reported for 17β-NT on human subjects [5,8,9,17–19,21] but also on horses [20], pigs and bovine animals [22–25], in particular calves [22–29]. In horses [30–32], main reported metabolites observed after 17β-NT administrations are 17α-nortestosterone (17α-NT), 19-noretiocholanolone (NE) but also 19-norandrosterone (NA), which has rarely been observed in cattle. These metabolic pathways are slightly different from that observed in human [5,8,17,20,21] and pig [32–34] where no epimerization exists and main observed metabolites are NA and NE. In cattle, the major pathway is C17 epimerization, 17α-NT being the most abundant metabolite after 17β-NT administrations [22–25,29,35]. Most of the previous studies have also reported, in different animal species, estranediols as important metabolites resulting from the complete reduction of Δ4-3-oxo group. In horses, the isomers 5α-estrane-3β,17α-diol (*aba*) and 5α-estrane-3β,17β-diol (*abb*) have been detected as main metabolites [30,31]. In bovines, the configuration 5α-estrane-3β,17α-diol, *aba*, has been identified as the predominant one [22,23,25,36]. The isomers *bab* and *abb* have been identified in pigs after 17β-nandrolone laureate ester administration [32–34]. However, only few of these studies have reported quantitative data on 17β-NT metabolites. In a previous paper [29], we reported elimination kinetic in calves' urine of 17β-nortestosterone laureate

☆ Article from special issue on "Steroid profiling and analytics: going towards Sterome".

* Corresponding author. Tel.: +33 2 40 68 78 80; fax: +33 2 40 68 78 78.
E-mail address: pinel@vet-nantes.fr (G. Pinel).
[1] laberca@vet-nantes.fr.

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.01.012

ester after its administration, by monitoring 17β-NT and its main metabolites: 17α-NT, NE, NA and 19-norandrostenedione (NAED). The purpose of the present study was to investigate further the metabolism of 17β-NT after 17β-nortestosterone laureate ester administration through estranediols profiling in urine samples. In this context, five isomers were selected and monitored including 5α-estrane-3β,17β-diol (*abb*), 5β-estrane-3α,17β-diol (*bab*), 5α-estrane-3β,17α-diol (*aba*), 5α-estrane-3α,17β-diol (*aab*) and 5β-estrane-3α,17α-diol (*baa*).

2. Materials and methods

2.1. Reagents and chemicals

Most of the reagents and solvents were of analytical grade quality and provided by Carlo Erba Réactifs SDS (Val de Rueil, France). Envi-ChromP and silica (0.5 g and 1 g stationary phase, respectively) solid-phase extraction (SPE) cartridges were purchased from Carlo Erba Réactifs SDS (Val de Rueil, France). Derivatisation reagents N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), dithiothreitol (DTE) and trimethyliodosilane (TMIS) were purchased from Sigma–Aldrich (St. Quentin Fallavier, France). The reference steroids were from Interchim (Montluçon, France), Sigma–Aldrich (St. Quentin Fallavier, France), AGAL/NARL (Australia) and RIVM (Bilthoven, The Netherlands). The internal standards used were 17β-nandrolone-d_3 (17β-NT-d_3), 19-norandrosterone-d_4 (NA-d_4) and 19-noretiocholanolone-d_3 (NE-d_3).

2.2. Animal experiment

An animal experiment has been conducted within the department of Veterinary Animal Health of the Faculty of Veterinary Medicine of the Utrecht University (The Netherlands). The protocol has been approved by the ethical committee from Utrecht University [29,37]. Twenty-four calves (Breed: Holstein-Friesen × Fries-Holland), twelve male and twelve female animals, were obtained from identified sources at an age of two weeks (10 days minimum and 3 weeks maximum) and subjected to a seven-week acclimatization period. Twelve healthy calves (6 females and 6 males) were selected randomly for the study after the acclimatization period and assigned to the treatment group. At the same time, another 12 healthy calves (6 females and 6 males) were selected randomly and assigned to the control group.

Over the study period, each animal from the treatment group received three intramuscular doses of a mixture of 5 mL 17β-oestradiol benzoate (Oestradiol Benzoaat®, 5 mg/mL, Intervet, Boxmeer, The Netherlands) and 3 mL 17β-nortestosterone laureate (Decadurabolin®, 50 mg/mL, Intervet, Boxmeer, The Netherlands) at day 0, day 14 and day 28. The first dose was administered at the average age of 10 weeks. Urine samples were collected at regular time points (20 and 11 collection points for treated and non treated calves, respectively) starting seven days before administration (average age 9 weeks) and stored prior to analysis below −18 °C. At day 42 (average age 16 weeks) treated animals were euthanized.

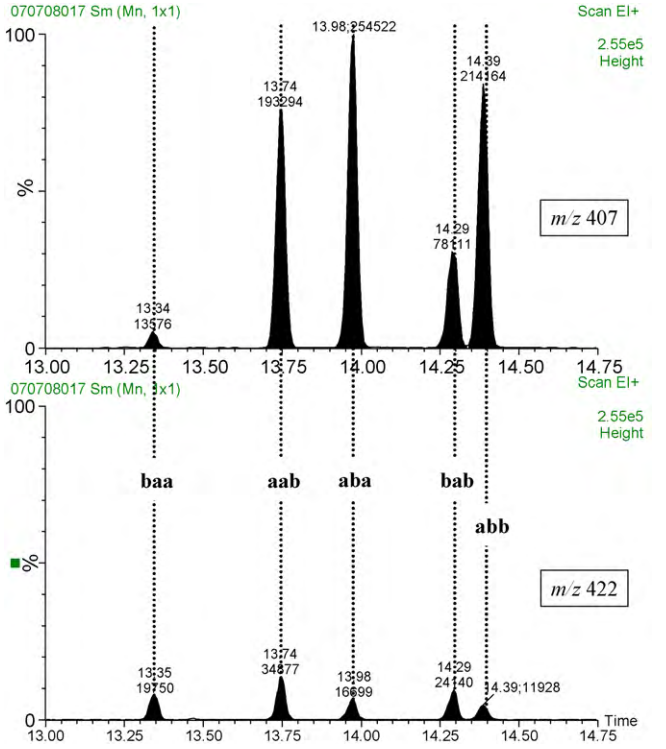

Fig. 1. Chromatograms for the ions *m/z* 422 and 407, corresponding to the molecular ions and demethylated compounds of derivatised *baa*, *aab*, *aba*, *bab* and *abb* (standard sample; 5 ng per compound injected). GC–EI(+)–MS analysis and SCAN acquisition mode.

2.3. Extraction and purification procedure

Aliquots of urine samples (10 mL) were added with 10 ng 17β-NT-d_3, NA-d_4 and NE-d_3, 1 mL acetate buffer (2 M, pH 5.2) and 200 μL β-glucuronidase from *Helix pomatia*. Hydrolysis was performed over 15 h at 52 °C. Urine samples were centrifuged (10 min, 1000 × *g*) before purification onto SPE Envi-ChromP. Cartridges were conditioned with 6 mL ethyl acetate, 6 mL methanol then 6 mL water. The extract was applied onto the column. The phase was washed with 3 mL water then 2 mL hexane. High vacuum was applied before and after each washing. Steroids were eluted with 14 mL hexane/diethylether (70:30; v/v) which were evaporated to dryness under a gentle stream of nitrogen. After hydrolysis, 1 mL NaOH 1N was added. Liquid/liquid extractions in alkaline medium phase performed with 4 mL hexane/diethylether (70:30; v/v) permitted to extract nortestosterone's metabolites.

After evaporation, the dry residue was dissolved in 500 μL hexane/dichloromethane (60:40; v/v) and applied onto a SPE sil-

Table 1
Diagnostic signals and spectrometric conditions used for monitoring each target estranediols of interest and internal or external standards.

Molecules	Transition 1	Collision T1 (eV)	Transition 2	Collision T2 (eV)	Transition 3	Collision T3 (eV)	Transition 4	Collision T4 (eV)	T_R (min)
baa	422.4 > 242.2	10	407.4 > 241.2	12	422.4 > 332.3	10	422.4 > 201.2	25	14.18
Norandrosterone-d_4	424.4 > 409.4	12							14.35
aab	407.4 > 241.2	12	407.4 > 199.1	20	407.4 > 145.1	30	422.4 > 242.2	10	14.63
aba	407.4 > 241.2	12	407.4 > 199.1	20	407.4 > 145.1	30	422.4 > 242.2	10	14.87
Noretiocholanolone-d_3	423.4 > 408.4	12							14.9
bab	407.4 > 241.2	12	422.4 > 242.2	10	407.4 > 145.1	30	407.4 > 331.3	10	15.20
abb	407.4 > 241.2	12	407.4 > 199.1	20	407.4 > 145.1	30	407.4 > 331.3	10	15.31
17β-Nandrolone-d_3	421.4 > 194.1	20							16.35
Norgestrel	456.4 > 301.3	25							18.76

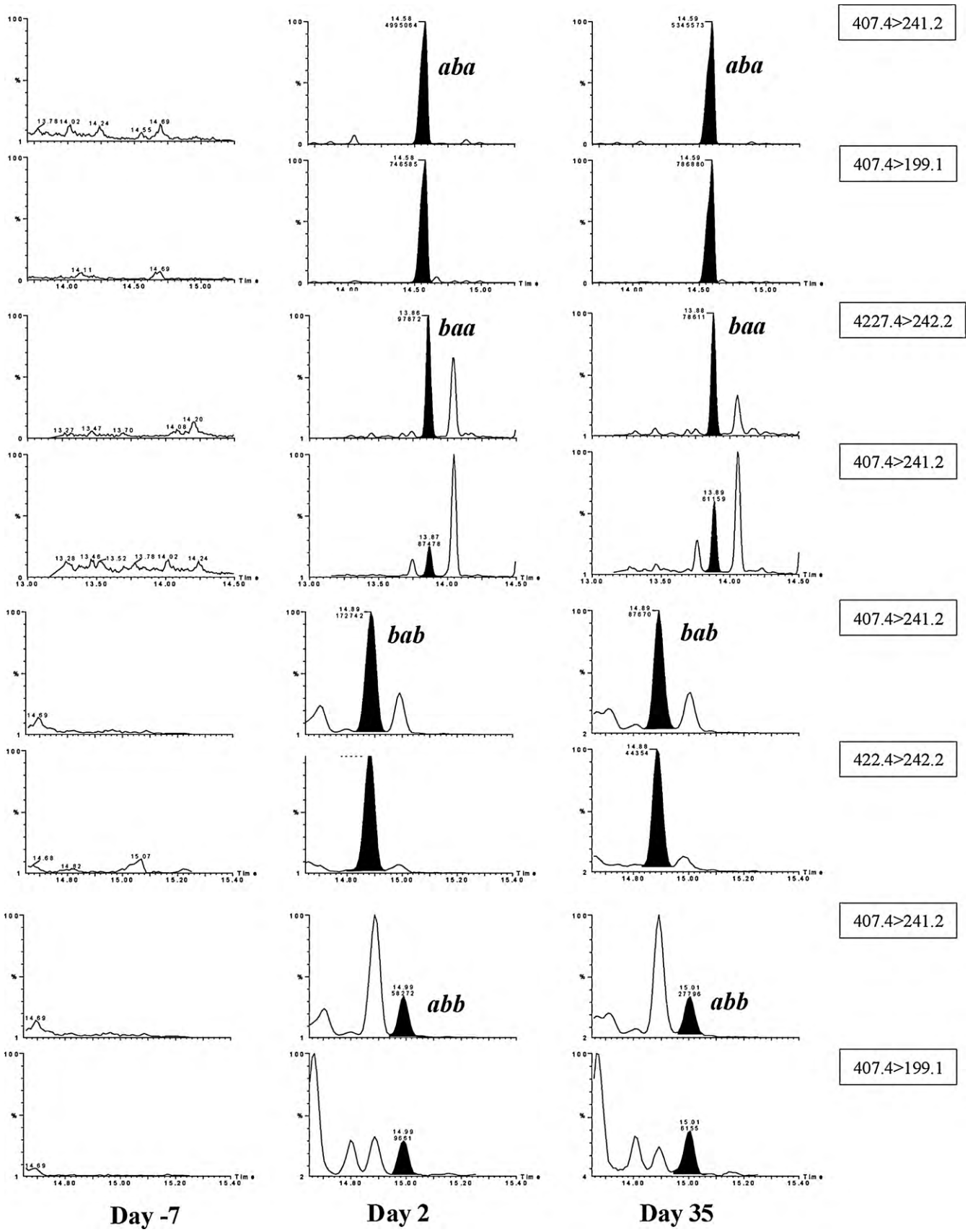

Fig. 2. Example, for one calf, of ion chromatograms of urine samples collected on three points: before treatment (day −7), after the first administration (day 2) and at the end of the experiment (day 35). Monitored signals correspond to *aba*, *baa*, *bab* and *abb*. GC–EI(+)–MS/MS analysis and SRM acquisition mode.

ica column conditioned with hexane. The column was washed with 3 mL hexane/ethylacetate (75:25; v/v) then 8 mL hexane/ethylacetate (85:15; v/v). Analytes were eluted with 20 mL hexane/ethylacetate (60:40; v/v) which were evaporated to dryness under a gentle stream of nitrogen. After addition of 7.5 ng norgestrel (Sigma–Aldrich) as external standard, the samples were derivatised 40 min at 60 °C with MSTFA-TMIS-DTE (1000:5:5; v/v/w). Of this extract, 2 μL was injected onto the GC-column.

2.4. Gas chromatography–mass spectrometry measurement

HP 6890 gas chromatograph was coupled to a VG Quattro II (Micromass, Manchester, UK) triple quadrupole mass spectrometer and a OV-1 (OHIO VALLEY, 30 m × 0.25 mm, 0.25 μm) was used. Helium was used as carrier gas at a flow rate of 1 mL/min in the constant pressure mode and the transfer line was maintained at 320 °C. Pulsed splitless injection was operated at 250 °C and 60 kPa during 1 min. The initial oven temperature was 120 °C for 2 min and increased to 250 °C at 15 °C/min then to 300 °C at 5 °C/min and hold for 8 min. The mass spectrometer was operated in the selected reaction monitoring (SRM) acquisition mode after electron ionisation of the analytes. In the collision cell, argon was used as collision gas at 4.5×10^{-4} mbar. Table 1 summarizes the retention times (T_R values), and optimised acquisition parameters for transitions and collision energies for each compound of interest.

3. Results and discussion

For the purpose of the present work, a GC–MS/MS detection method was developed in order to separate and identify unambiguously the estranediol isomers: 5α-estrane-3β,17β-diol (*abb*), 5β-estrane-3α,17β-diol (*bab*), 5α-estrane-3β,17α-diol (*aba*), 5α-estrane-3α,17β-diol (*aab*) and 5β-estrane-3α,17α-diol (*baa*). As shown in Fig. 1, the extracted ion chromatograms of the 5 derivatised compounds of interest acquired in full scan mode and corresponding to the molecular ions (*m/z* 422) as well as their demethylated forms (*m/z* 407) give evidence for their good chromatographic separation which enables individual monitoring.

Urines samples collected on 6 treated calves (3 males and 3 females) taken during the whole time window of the experiment (on days −7, 0, 1, 2, 3, 4, 14, 15, 16, 17, 18, 28, 29, 30, 31, 32 and 35) and corresponding to 102 samples, have been analysed in the present study. For each of these compounds, except for *aab* which has never been identified in the samples, Fig. 2 shows 2 ions chromatograms corresponding to 3 collection points: before administration (day −7), after the first administration (day 2) and at the end of the experiment (day 35). When none of the monitored compounds were detected before administration, they could be identified in urine samples of treated animals immediately after the first injection and during all the experiment. If *aba* has already been described in literature as a urinary metabolite of 17β-NT in cattle [22], the present study also allowed detection of additional estranediol isomers, namely *baa*, *bab* and *abb*, which have never been reported in bovines previously. For each treated calves and for each isomer, kinetics of elimination are shown in Fig. 3 it appears that whatever the considered estranediol and the animal involved in the study, profiles of elimination are similar. No significant difference could be observed between male and female animals in term of global urinary excretions profiles whereas some important differences in terms of concentration range could be observed for *aba*, *baa* and *abb*, which were respectively excreted in average 2, 1.5 and 1.5 times higher in female urines than in male samples.

After each injection of 17β-nortestosterone laureate ester, the urinary elimination of estranediols increases but not proportionally

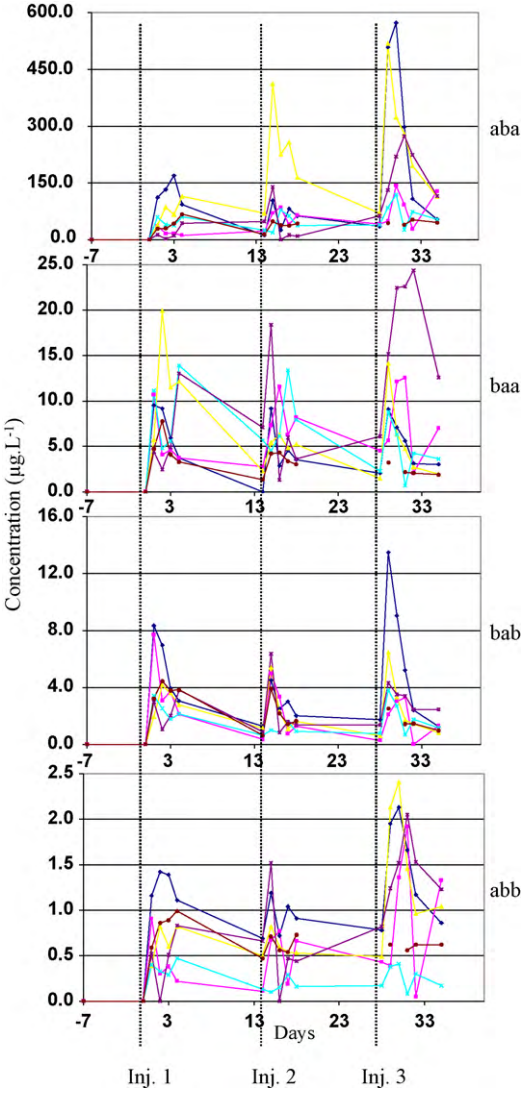

Fig. 3. Kinetics of elimination over 35 days of *aba*, *baa*, *bab* and *abb* for 6 17β-NT laureate/17β-E2 benzoate treated calves. Concentrations have been calculated from GC–EI(+)–MS/MS analysis of collected urine samples.

to the injected dose. The excretion rates reach the highest value 24–72 h after each administration. Respective concentrations are different depending on the metabolite considered, *aba* exhibiting the highest concentration level (>100 μg L^{-1}) whereas *baa*, *bab* and *abb* mean highest concentration values were 15, 7 and 1.6 μg L^{-1}, respectively. These results are in agreement with the metabolism of 17β-NT in bovine species: since C17 epimerization is a major pathway, the urinary concentration of 17α metabolites is expected more important than that of 17β-estranediols (Fig. 4). Amongst the monitored estranediols, the most important one, in term of urinary concentrations, is the 5α-estrane-3β,17α-diol (*aba*), which is in agreement with previous studies [22,23,25] reporting this metabolite as the most abundant HO-catabolite of 17β-NT in cattle.

The present results combined with those from the previous study [29] allow concluding on the privileged metabolism pathway for 17β-NT in calves with predominance of the 17α-metabolites 17α-NT then *aba*. Since it is generally accepted that 17α-NT is the major metabolite in urine, this analyte is the target residue in most residue control plan. However, as presented by Daeseleire et al. [22] this is not always the case: the predominance of *aba* in calves' urines after oral intake of 17β-NT was reported whereas after intramuscular injection of 17β-NT laureate the most abundant metabolite

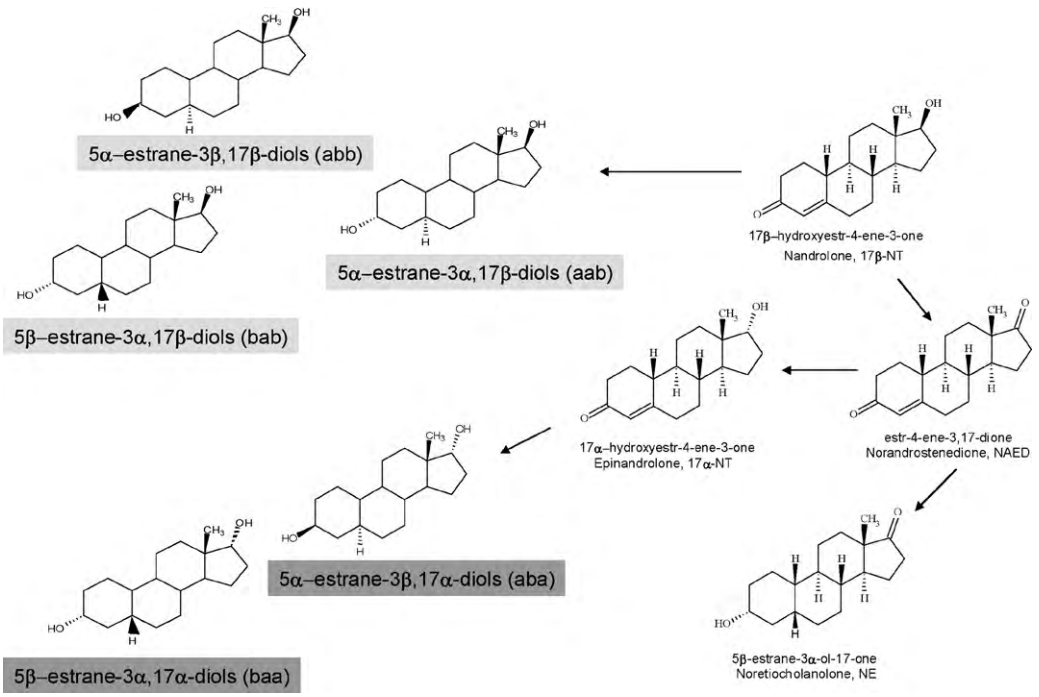

Fig. 4. Proposition of nandrolone (17β-NT) metabolic pathway.

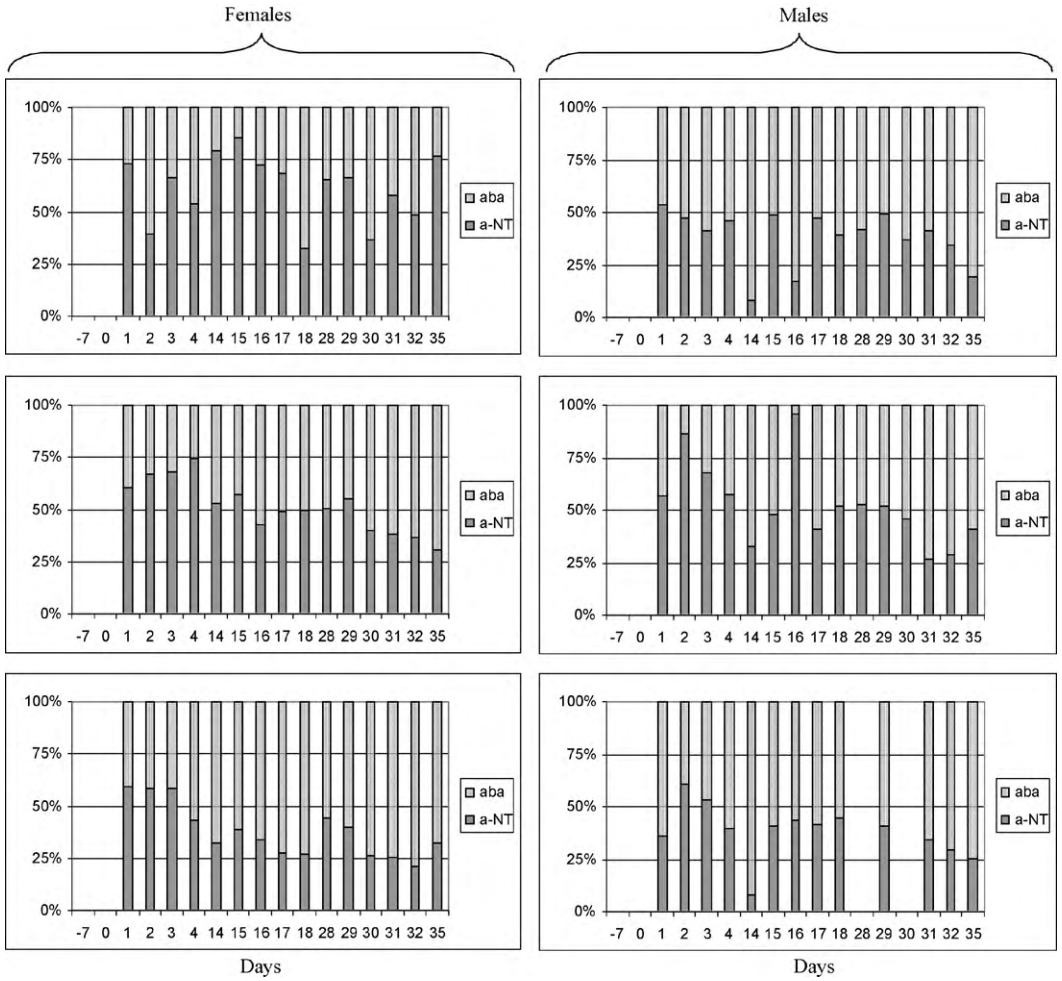

Fig. 5. Kinetic profiles of the ratio between the 2 major monitored metabolites, *aba* and 17α-NT, over 35 days and for 6 17β-NT laureate/17β-E2 benzoate treated calves. Percentages have been calculated from GC–EI(+)–MS/MS data analysis of collected urine samples.

was 17α-NT. Fig. 5 presents urinary concentrations ratios between the two major metabolites found in the present study, *aba* and 17α-NT, along the experiment. For each animal, no significant difference could be observed between males and females in term of kinetic profile. Theses ratios indicate that *aba* and 17α-NT are excreted in similar concentrations, and in quite equivalent proportions. Both of them can be considered as major metabolite of 17β-NT in calves.

4. Conclusion

Focussing on the particular estranediols steroid fraction, the present work allowed further investigation of 17β-NT metabolism in calves after 17β-NT laureate ester administration. A GC–MS/MS method for the detection and identification of five estranediol isomers (*aba*, *baa*, *bab*, *abb* and *aab*) has been set up. The application of the method to the analysis of urine samples collected on treated animals revealed that 4 of the them (*aba*, *baa*, *bab*, and *abb*) could be detected in the samples after 17β-NT laureate ester administration, *aba* being the major one. Elimination profiles have been established allowing detection of the compounds up to several days after 17β-NT administration which may open a new way for the control of anabolic steroid administration by global steroid profiling and therefore extend the field of investigation. Indeed and mainly as a result of this extensive metabolism combined with the possibility of interferences with other endogenous compounds, detection of the illegal use of 17β-NT often turns out to be a difficult issue. In recent years, proving the illegal administration of 17β-NT became even more challenging since different authors reported data to demonstrate the endogenous presence of 17β-NT or some of its metabolite in different species [19,38–41]. In bovines particularly, if it is known for a long time that 17α-NT can occur naturally in the urine of pregnant cows [42], it was generally accepted that neither 17α-NT nor 17β-NT occur naturally in the urine of steers or bulls [43]. Nevertheless, some recent findings reported that both forms can be detected in injured animals [44] which will most likely represent a difficulty for EU member states who currently rely on both forms measurements to monitor and control the abuse of this popular drug.

Acknowledgments

This work has been supported by the European Commission under the specific research and technological development of the 6th Framework Programme “Integrating and strengthening the European Research Area within the BioCop project “New technologies to Screen Multiple Chemical Contaminants in Foods”. Contract number: FOOD-CT-2004-06988.

We gratefully thank Dr. Suzanne WF. Eisenberg who was responsible for the animal experiment and coordination of the sample collection.

References

[1] Council Directive 96/22/EC, of 29 April 1996. Off. J. Eur. Commun. (1996) No L 125/3. Concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and on b-agonists and repealing Directives 81/602/EC, 88/146/EC and 88/299/EC.
[2] M. Dubois, X. Taillieu, Y. Colemonts, B. Lansival, J. De Graeve, P. Delahaut, MS determination of anabolic steroids after multi-immunoaffinity purification, Analyst 123 (1998) 2611–2616.
[3] S. Impens, K. De Wasch, H. De Brabander, Determination of anabolic steroids with gas chromatography-ion trap mass spectrometry using hydrogen as gas carrier, Rapid Commun. Mass Spectrom. 15 (2001) 2409–2414.
[4] D. Catlin, B. Ahrens, Y. Kucherova, Detection of norbothenone an anabolic steroid never marked in athletes’ urine, Rapid Commun. Mass Spectrom. 16 (2002) 1273–1275.
[5] D. Thieme, P. Anielski, J. Grosse, H. Sachs, R.K. Mueller, Identification of anabolic steroids in urine, serum sweat and hair—comparison of metabolic pattern, Anal. Chim. Acta 483 (2003) 299–306.
[6] J. Munoz-Guerra, D. Carreras, C. Soriano, C. Rodriguez, A.F. Rodriguez, Use of ion trap gas chromatography–tandem mass spectrometry for detection and confirmation of anabolic substances at trace level in doping analysis, J. Chromatogr. B 704 (1997) 129–141.
[7] Y. Gaillard, F. Vayssette, A. Balland, G. Pépin, Gas chromatographic–tandem mass spectrometric determination of anabolic steroids and their esters in hair: application in doping control and meat quality control, J. Chromatogr. B 735 (1999) 189–205.
[8] B. Le Bizec, F. Bryand, I. Gaudin, F. Monteau, F. Poulain, F. André, Preliminary results to discriminate between endogenous and exogenous origin, Steroids 67 (2002) 105–110.
[9] P. Van Eenoo, F.T. Delbeke, F.H. de Jong, P. De Backer, Endogenous origin of norandrosterone in female urine: indirect evidence for the production of 19-norsteroids as by-products in the conversion from androgen to estrogen, J. Steroid Biochem. Mol. Bio. 78 (2001) 351–357.
[10] C. Van Poucke, C. Van Peteghem, Development and validation of a multi-analyte method for detection of anabolic steroids in bovine urine with liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 772 (2002) 211–217.
[11] C. Van, M. Poucke, C. Van De Velde, Van Peteghem, Combination of liquid chromatography/tandem mass spectrometry and gas chromatograph/mass spectrometry for the detection of 21 anabolic steroid residues in bovine urine, J. Mass Spectrom. 40 (2005) 731–738.
[12] M.W.F. Nielen, P. Rutgers, E.O. Van Bennekom, J.J.P. Lasaroms, J.A. Van Rhijn, Confirmatory analysis of 17β-boldenone, 17α-boldenone, and androsta-1,4-diene-3,17-dione in bovine urine, faeces, feed and skin swab samples by liquid chromatography–electrospray ionisation tandem mass spectrometry, J. Chromatogr. B 801 (2004) 273–283.
[13] A. Leinonen, T. Kuuranne, T. Kotiaho, R. Kostiainen, Screening of free 17-alkyl-substituted anabolic steroids in human urine by liquid chromatography–electrospray ionization tandem mass spectrometry, Steroids 69 (2004) 101–109.
[14] R. Draisci, L. Palleschi, E. Ferreti, L. Lucentini, P. Cammarata, Quantitation of anabolic hormones and their metabolites in bovine serum and urine by liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 870 (2000) 511–522.
[15] M.W.F. Nielen, J.J.P. Lasaroms, M.L. Essers, M.B. Sanders, H.H. Heskamp, T.F.H. Bovee, J.H. van Rhijn, M.J. Groot, Multi-residue screening of intact testosterone esters and boldenone undecylenate in bovine hair using liquid chromatography electrospray tandem mass spectrometry, Anal. Chim. Acta 830 (2006) 126–134.
[16] D. Mottram, A. George, Anabolic steroids, Clin. Endocrinol. Metab. 14 (2000) 55.
[17] B. Le Bizec, I. Gaudin, F. Monteau, F. André, S. Impens, K. De Wasch, H. De Brabander, Consequence of boar edible tissue consumption on urinary profiles of nandrolone metabolites. I. mass spectrometric detection and quantification of 19-norandrosterone and 19-noretiocholanolone in human urine, Rapid Commun. Mass Spectrom. 14 (2000) 1058–1065.
[18] Y.L. Tseng, C.-Y. Sun, F.-H. Kuo, Detection and quantification of glucuro- and sulfoconjugated metabolites in human urine following oral administration of xenobiotic 19 norsteroids, Steroids 71 (2006) 817–827.
[19] B. Le Bizec, F. Monteau, I. Gaudin, F. André, Evidence for the presence of endogenous 19-norandrosterone in human urine, J. Chromatogr. B 723 (1999) 157–172.
[20] L. Dehennin, Y. Bonnaire, Ph. Plou, Human nutritional supplements in horse: comparative effects of 19-norandrostenediol on the 19-norsteroid profile and consequences for doping control, J. Chromatogr. B 766 (2002) 287–294.
[21] R. Massé, C. Ayotte, R. Dugal, Integrated methodological approach to GC–MS analysis of anabolic steroid metabolites in urine, J. Chromatogr. 489 (1989) 23–50.
[22] E. Daeseleire, A. De Guesquière, C. Van Peteghem, Metabolism of 1-19-nortestosterone in urine of calves after oral intake and intramuscular administration, Anal. Chim. Acta 275 (1993) 95–103.
[23] E. Benoît, J.L. Guyot, D. Courtot, P. Delatour, Gas chromatographic/mass spectrometric analysis of 19-nortestosterone urinary metabolites in cattle, Ann. Rec. Vet. 20 (1989) 485–491.
[24] S. Calvarese, P. Rubini, G. Urbani, N. Ferri, C. Ramazza, M. Zucchi, Experimental administration of 19-nortestosterone and dexamethasone in cattle: elimination of the two drugs in different biological matrices, Analyst 119 (1994) 2611–2615.
[25] E. Benoit, E. Champelle, D. Courtot, P. Delatour, Dépistage de l’utilisation de la nandrolone en élevage bovin, Rec. Med. Vet. 165 (1989) 737–740.
[26] W. Haasnoot, R. Shilt, A.R.M. Hamers, F.A. Huf, Determination of b-19-nortestosterone in biological samples at the sub parts per billion level by high-performance liquid chromatography with on-line immunoaffinity sample pretreatment, J. Chromatogr. 489 (1989) 157–171.
[27] Van Ginkel, R.W. Stephany, H.J. Rossum, H. Van Blitterswijk, P.W. Zoontjes, R.C.M. Hooijschuur, J. Zuydendorp, Effective monitoring of residues of nortestosterone anf its major metabolite in bovine urine and bile, J. Chromatogr. 489 (1989) 95–104.
[28] H. Lu, G. Conneely, M.A. Crowe, M. Aherne, M. Pravda, G.G. Guilbault, Screening for testosterone, methyltesosterone, 19-nortestosterone residues and their metabolites in bovine urine with enzyme-linked immunosorbent assay (ELISA), Anal. Chim. Acta 570 (2006) 116–123.
[29] G. Pinel, L. Rambaud, G. Cacciatore, A. Bergwerff, C. Elliot, M. Nielen, B. Le Bizec, Elimination kinetic of 17β-estradiol 3-benzoate and 17β-nandrolone laureate ester metabolites in calves’ urine, J. Steroid Biochem. Mol. Bio. 110 (2008) 30–38.

[30] E. Houghton, Studies related to metabolism of anabolic steroids in the horse: 19-nortestosterone, Xenobiotica 7 (1977) 683–693.
[31] E. Houghton, M.C. Dumasia, Studies related to the metabolism of anabolic steroids in the horse: the identification of some 16 oxygenated metabolites of 19-nortestosterone, Xenobiotica 10 (1980) 381–389.
[32] M. Roig, J. Segura, R. Ventura, Quantitation of 17β-nandrolone metabolites in boar and horse urine by gas chromatography–mass spectrometry, Anal. Chim. Acta 586 (2007) 184–195.
[33] G. Debruyckere, C. Van Peteghem, H.F. De Brabander, G. Debackere, Gas chromatographic–mass spectrometric confirmation of 19-nortestosterone in the urine of untreated boars—effect of the administration of Laurabolin, Vet. Quart. 12 (1990) 246–251.
[34] G. Debruyckere, C. Van Peteghem, Detection of 19-nortestosterone and its urinary metabolites in miniature pigs by gas-chromatography–mass spectrometry, J. Chromatogr. 564 (1991) 393–403.
[35] J. Scarth, C. Ackre, L. Van Ginkel, B. Le Bizec, H. De Brabander, W. Korth, J. Points, P. Teale, J. Kay, Presence and metabolism of endogenous androgenic–anabolic steroid hormones in meat producing animals: a review, Food Additives Contam. 26 (2009) 640–671.
[36] T. Tuinstra, P. Kienhuis, R.G. Van Mazijk, W. Traag, Proc. Int. Symp. of Veterinary Laboratory Diagnosticans, Amsterdam, 1986, pp. 765–767.
[37] S.W.F. Eisenberg, G. Cacciatore, S. Klarenbeek, A.A. Bergwerff, A.P. Koets, Influence of 17β-oestradiol, nortestosterone and dexamethasone on the adaptive immune response in veal calves, Res. Vet. Sci. 84 (2008) 199–205.
[38] S. Poelmans, K. De Wasch, H. Noppe, N. Van Hoof, S. Van Cruchten, B. Le Bizec, Y. Deceuninck, S. Sterck, H.J. Van Rossum, M.K. Hoffman, H.F. De Brabander, Endogenous occurrence of some anabolic steroids in swine matrices, Food Additives Contam. 22 (2005) 808–815.
[39] J. Rosegger, I. Schmerold, S. Ahmed, R. Schuch, G. Eppinger, S. Steiner, W. Baumgartner, H. Armstrong, G. Schauberger, J.D.G. McEvoy, T.W. Kuhn, Natural occurence and elimination of 19-nortestosterone in sheep: pregnant ewes, male and female lambs before and after treatment, Wiener Tierarztliche Monatsschrift 96 (2009) 171–183.
[40] H. De Brabander, J. Van Hende, P. Batjoens, L. Hendricks, J. Raus, F. Smet, G. Pottie, L. Van Ginkel, R.W. Stephany, Endogenic nortestosterone in cattle? Analyst 119 (1994) 2581–2585.
[41] M. Vandenbroeck, G. Van Vyncht, G. Gaspar, C. Dasnois, P. Delahaut, G. Pelzer, J. De Graeve, G. Maghuin-Rogister, Identification and characterization of 19-nortestosterone in urine of meat-producing animals, J. Chromatogr. 564 (1991) 405–412.
[42] H. Meyer, D. Falckenberg, T. Janowski, M. Rapp, E.F. Rösel, L. Van Look, H. Karg, Acta Endocrinol. 126 (1992) 369–373.
[43] J.D.G. McEvoy, W.J. McCaughey, J. Cooper, D.G. Kennedy, B.M. McCarten, Nortestosterone is not a naturally occurring compound in male cattle, Vet. Quart. 21 (1999) 8–15.
[44] D.G. Kennedy, H.D. Shortt, S.R.H. Crooks, P.B. Young, H.J. Price, W.G. Smyth, S.A. Hewitt, The occurrence of α and β-nortestosterone residues In the urine of injured male cattle, in: Proceedings from Euroresidue VI Conference, 19–21 May, Egmond An Zee, The Netherlands, 2008.