

# Gas chromatographic-mass spectrometric confirmation of 19-nortestosterone in the urine of untreated boars — effect of the administration of Laurabolin®

G. Debruyckere<sup>1</sup>, C. Van Peteghem<sup>1</sup>, H. F. De Brabander<sup>2</sup>, and M. Debackere<sup>3</sup>

**SUMMARY** *The presence of 17 $\beta$ -19-nortestosterone (nandrolone, NT, 17 $\beta$ -19-NT) and its epimer 17 $\alpha$ -19-nortestosterone (epiNT, 17 $\alpha$ -19-NT) was investigated in the urine of six untreated boars, obtained from experimental farms. The presence of 17 $\beta$ -19-nortestosterone was screened by RIA and HPTLC and confirmed by GC-MS analysis. Additionally, the two epimers (NT and epiNT) were investigated in the urine of a boar (two-year-old miniature male pig weighing 50 kg) before and after injection of 100 mg Laurabolin® (nortestosterone laurate, Intervet N.V., Belgium).*

*The isolation of the steroids was based on sample clean-up with solid phase extraction and subsequent high-performance liquid chromatography. Both gas chromatographic retention data and mass spectrometric data (selected ion monitoring and full spectrum) were used for detection and identification.*

*The presence of 17 $\beta$ -19-nortestosterone in the urine of the boars that were not injected proves the endogenous production of the steroid. The absence of the 17 $\alpha$ -epimer in the urine of the injected boar suggests that 17 $\alpha$ -19-nortestosterone is not a major metabolite of 17 $\beta$ -19-nortestosterone.*

<sup>1</sup> Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, State University of Ghent, Belgium.

<sup>2</sup> Laboratory of Chemical Analysis of Food from Animal Origin, Faculty of Veterinary Medicine, State University of Ghent, Belgium.

<sup>3</sup> Laboratory of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, State University of Ghent, Belgium.

All correspondence should be addressed to: Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, Harelbekestraat 72, 9000 Ghent, Belgium.

## INTRODUCTION

The illegal use of anabolic steroids in livestock breeding has taken on enormous proportions in the last few decades. Measures are taken by the health authorities to protect the consumer from possible harmful effects due to the consumption of contaminated meat or meat products. Detection of the illegal use of anabolics is difficult as a result of the extensive metabolism of steroid hormones and the possibility of interference with endogenous steroids, which are species dependent. Nandrolone (17 $\beta$ -19-nortestosterone, NT, 17 $\beta$ -19-NT) is a frequently used anabolic steroid in cattle fattening. However, 19-nortestosterone has also been detected as a natural steroid in stallion urine (8, 9), it has been identified in the follicular fluid of the mare (4, 13) and its secretion by the testis of the stallion (1, 5) and the boar (11) has been evidenced. In human follicular fluid 19-nortestosterone has also been identified (3, 4). Only some of the above references specify the 17 $\beta$ -epimeric form of 19-nortestosterone.

Nandrolone has also been detected and quantified in edible parts of non-castrated male pigs, in particular in the liver and muscle tissue (15). Besides boar taint, which is due to the presence of androstenone (5 $\alpha$ -androst-16-en-3-one), the occurrence of 17 $\beta$ -19-nortestosterone as a natural constituent of boar meat produces a second 'steroid problem' in pork trade in Europe. Moreover, the tendency to reduce boar castration in the EEC will increase these problems further. Nandrolone will become a more likely natural constituent of pork and meat products derived from pork. Recently, 17 $\beta$ -19-NT was detected in ravioli imported from Italy into Germany. Since the pork used in these products could be originating from non-castrated boars, 17 $\beta$ -19-NT should be considered to be natural in ravioli. This is extremely important for food inspection services.

During regulatory control in the slaughterhouse, it was frequently observed that boar urine shows a RIA-positive result on 19-nortestosterone. The presence of 17 $\beta$ -19-NT in these urine samples was already presumed by one of the authors and later confirmed by some Belgian laboratories involved in the control for the Institute of Veterinary Inspection (10, Van Peteghem C, De Brabander HF, Smits F, Pottie G. Unpublished results). In order to demonstrate that residues could be due to endogenous production, urine samples of clean boars ( $n = 6$ ) from experimental farms were analysed. Additionally, the presence of 17 $\beta$ -19-nortestosterone and its epimer 17 $\alpha$ -19-nortestosterone (epiNT, 17 $\alpha$ -19-NT) was traced in the urine of a two-year-old miniature male pig after treatment with Laurabolin® (nortestosterone laurate).

## MATERIALS AND METHODS

### *Procedure*

In the present study, gas chromatography-mass spectrometry (GC-MS) was used for the confirmation of 17 $\beta$ -19-NT after the preliminary radioimmunoassay (RIA) (6) and high-performance thin-layer chromatography (HPTLC) (14) screening tests.

### *Animals*

Urine samples were obtained from six untreated boars (Belgian Landrace), obtained from experimental farms, having an age between two and three years and weighing between 150 and 200 kg. A two-year-old miniature boar (75% Göttinger, 25% Vietnamese) of 50 kg was treated with Laurabolin® (100 mg nortestosterone laurate, Intervet N.V. Belgium). Urine samples were collected before and at regular intervals after Laurabolin® treatment.

### *Instrumentation*

Solid-phase extraction for sample preparation was carried out using octadecyl (C<sub>18</sub>) and amino (NH<sub>2</sub>) disposable columns (J. T. Baker, Phillipsburg, New Jersey, USA).

High-performance liquid chromatography (HPLC) was performed using a Waters pump (model 6000 A, Waters Associates, Milford, Massachusetts, USA) equipped with an automatic injector (WISP 710 B, Waters Associates, Milford, Massachusetts, USA) and a variable wavelength detector (model SP 8400, Spectra Physics, Santa Clara, California, USA). The HPLC analytical column used was a LiChrospher® 100 RP — 18 (5  $\mu$ m) column (Merck, Darmstadt, FRG) and was protected by a guard column (75 mm x 2.1 mm, cat. no. 28603, Chrompack, Middelburg, The Netherlands).

Gas chromatographic-mass spectrometric analyses were carried out on a HP-5890 gas chromatograph equipped with a 25 m HP Ultra 2 (Cross-Linked 5% Phenyl Methyl Silicone — 25 m x 0.2 mm I.D. x 0.33  $\mu$ m film thickness) fused silica capillary column and linked to a HP-5970 mass-selective detector (Hewlett-Packard, Palo Alto, California, USA).

### Sample preparation

Steroid extraction was based on the procedure of Schmidt *et al.* (12).

Free and conjugated steroids, contained in 10 mL of urine, were extracted on a C<sub>18</sub>-column. The conjugated steroids were hydrolysed by means of Helix pomatia digestive juice (Boehringer, Mannheim, FRG) at 37°C for 16 hours. The steroids were subsequently extracted on a C<sub>18</sub>-column. Additional purification was done by placing an amino column (NH<sub>2</sub>) in series with the C<sub>18</sub>-column.

The extract was finally fractionated by means of HPLC (mobile phase CH<sub>3</sub>OH/H<sub>2</sub>O: 65/35 at a flow-rate of 1 mL/min). The fraction containing the two epimers (5—7.5 min) was collected and evaporated to dryness under nitrogen.

### GC-MS analysis

After derivatisation with heptafluorobutyric acid anhydride (HFBA, Macherey-Nagel, Düren, FRG), an aliquot was injected into the GC-MS instrument. The injection temperature and the transfer line temperature were 280°C. The oven temperature was programmed from 200 to 270°C at 5°C/min and was kept constant at 270°C for 14 minutes. The carrier gas was helium at a column head pressure of 80 kPa. Samples were injected in the split mode. The mass spectrometer was used in the electron impact mode.

### RESULTS AND DISCUSSION

During preliminary experiments, the presence of 17 $\beta$ -19-NT was demonstrated in the urine of the clean boars (n = 6) by RIA and HPTLC. These results were confirmed by GC-MS in the selected ion monitoring (SIM)-mode. Ion chromatograms of the molecular ion (m/z 666) and two fragment ions (m/z 453 and m/z 306) were recorded. For a positive identification, the three ions must appear simultaneously at the right retention time, expressed in methylene unit values, as derived from the retention data of the pure reference compounds. The methylene unit values of 17 $\alpha$ -19-NT-diHFB and 17 $\beta$ -19-NT-diHFB are 23.62 and 24.38, respectively. These results give evidence of the endogenous production of 17 $\beta$ -19-NT in boars. Due to the apparently high concentration at which 17 $\beta$ -19-NT occurs in boar urine, the mass-selective detector could be used in the SCAN-mode, so that a full mass spectrum and thus more scientific evidence can be obtained. As can be seen from Figure 1, the spectrum of the alleged 17 $\beta$ -19-NT-diHFB (top) is identical to that of the reference compound 17 $\beta$ -19-NT-diHFB (bottom). No 17 $\alpha$ -19-NT could be detected in the samples taken before Laurabolin® administration, nor could any be detected in the urine samples of the miniature boar taken 92 hours, one week, two weeks and three weeks after the injection of Laurabolin®. The method was validated by spiking urine samples with 17 $\alpha$ -19-NT in a concentration of 5 ppb. It proves that, in contrast to what is observed in cattle (2, 7, 16), C-17 epimerisation is not a major pathway of metabolism of 17 $\beta$ -19-NT in boars.

In the future, experiments will be set up to investigate 19-nortestosterone metabolites in castrated and female pigs and to expand the results using a larger population of experimental animals.

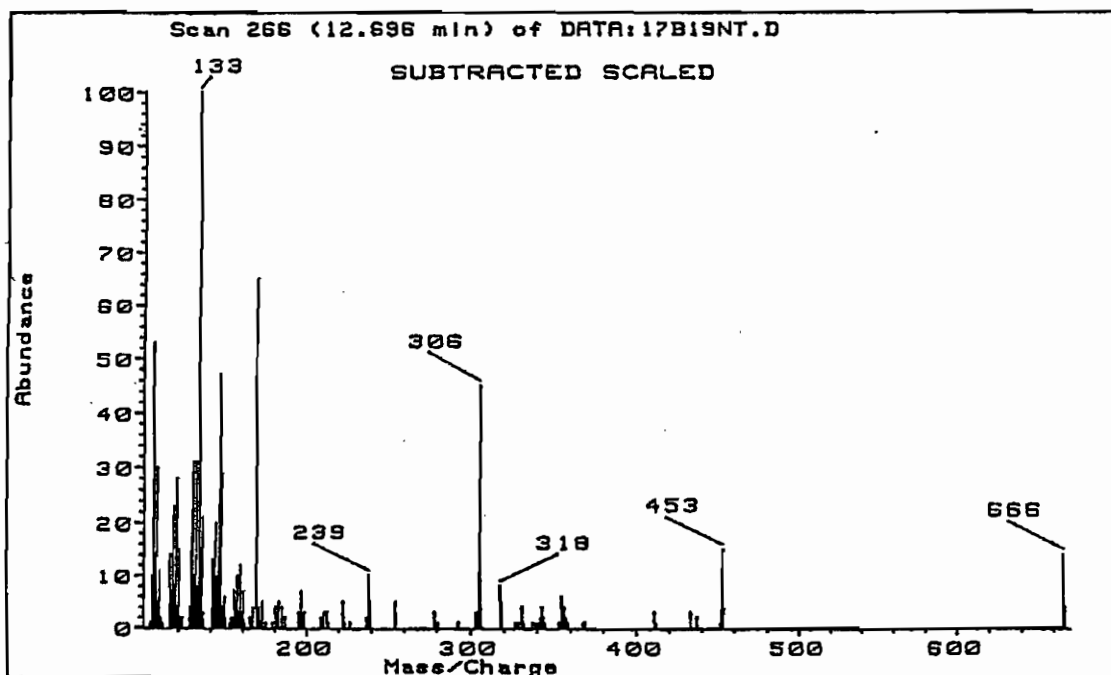
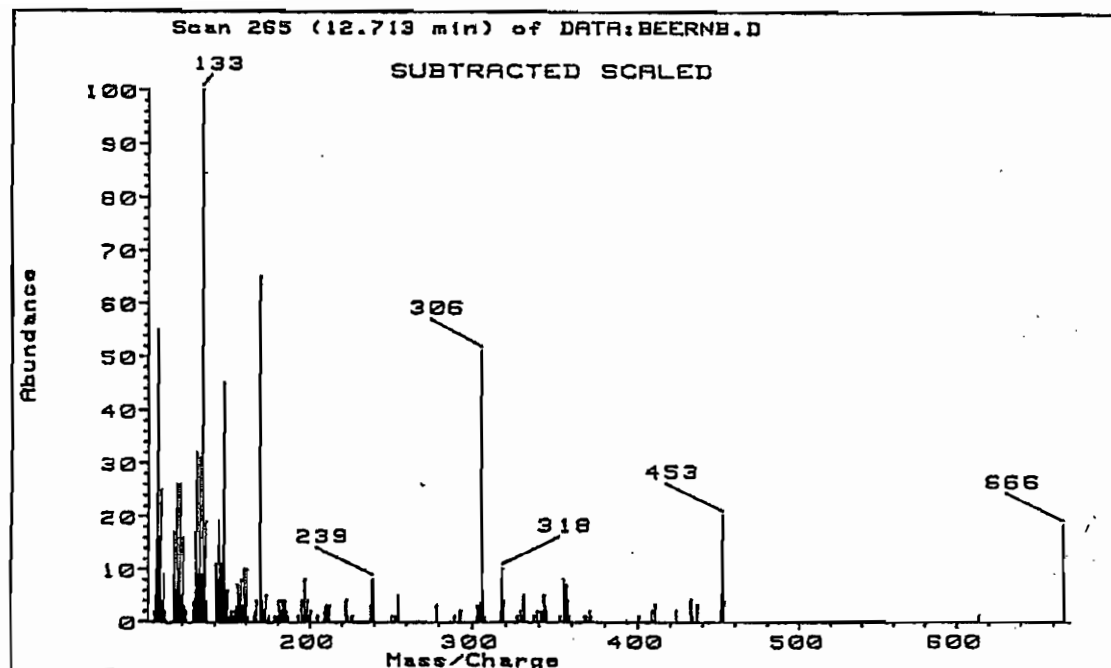


Figure 1. Mass spectrum of  $17\beta$ -19-NT-diHFB in the untreated boar (top) and of the reference  $17\beta$ -19-NT-diHFB (bottom).

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