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Identification and characterization of 19-nortestosterone in urine of meat-producing animals

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ABSTRACT

To monitor the illegal use of 19-nortestosterone as an anabolizing agent in meat-production, the Belgian Institute of Veterinary Expertise applies a strategy of urine control by radioimmunoassay, positive samples being confirmed by thin-layer chromatography. We have evaluated this control strategy, using gas chromatography–mass spectrometry to confirm the presence of 19-nortestosterone, or its metabolite oestrane-diol, in positive samples from radioimmunoassay. Our results show that the effective way of proceeding remains reliable in cattle, for mature and immature males as well as non-pregnant females, and in pigs, for pregnant and non-pregnant sows. The possible presence of endogenous 19-nortestosterone in cattle, in pregnant cows urine, and in pigs, in boars and in cryptorchid pigs, impedes the control of the use of 19-nortestosterone on these samples. False-positive (not confirmed by gas chromatography–mass spectrometry) results were produced by radioimmunoassay in the urine of castrated pigs and sheep.

INTRODUCTION

19-Nortestosterone (19-NT) is one of the main anabolics used illegally in cattle [1–3]. Therefore, the use of this steroid is monitored, in Belgium, by laboratories involved in the control of anabolics. The commonest technique used is to analyse urine samples by radioimmunoassay (RIA) [4].

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The presence of 2 ng/ml (ppb) and more of 19-NT in a urine sample leads, after confirmation by high-performance thin-layer chromatography (HPTLC), to the exclusion of the animal for consumption. However, this decision is taken only when the presence of either 17α- or 17β-19-NT is detected. The presence of a third substance, oestrane-diol, another metabolite of 17β-19-NT, has to be detected with 17α-19-NT or 17β-19-NT to confirm the use of the anabolic.

Recent studies have shown the existence of endogenous 19-NT in stallions and boars [5–7], as well as in mares and in pregnant women [8,9]. Moreover, the natural presence of the steroid has also been suspected in calves, because epitestosterone (present in high concentrations in these animals) is easily confused with 19-NT when HPTLC is used. These findings have cast doubt on the validity of the control strategy applied to the monitoring of the use of 19-NT in meat-producing animals.

Therefore, we have examined urine samples collected from untreated males (mature and immature) and females (pregnant and non-pregnant) from the three main meat-producing species (bovine, porcine and ovine) by RIA. The presence of 19-NT in the RIA-positive samples was confirmed by gas chromatography–mass spectrometry (GC–MS).

Indeed, RIA, even when highly specific and sensitive, has the disadvantage that cross-reactions may interfere in the interaction between the antibody and the antigen. Complementary sensitive and specific techniques are therefore required to detect residues of forbidden substances in biological samples. HPTLC is at best a semi-quantitative method. According to Stephany [10], the identification of a compound has to be based on spectrometric methods directly related to the molecular structure. GC–MS furnishes that kind of direct information and is, therefore, more reliable than methods that provide indirect information, such as rate factors in HPTLC or affinity for an antibody in RIA.

EXPERIMENTAL

Urine samples

Urine from untreated animals was collected from farms and slaughter-houses, from mature and immature males as well as pregnant and non-pregnant females from the three main meat-producing species (bovine, porcine and ovine).

Materials

Tritiated 19-NT (19 Ci/mmol) was obtained from Amersham (Little Chalfont, U.K.). The steroid standards 19-NT and methyltestosterone were purchased from Steraloids (Wilton, NH, U.S.A.), and β-glucuronidase–arylsulphatase from *Helix pomatia* was obtained from Boehringer Mannheim (Mannheim, Germany). Anti-19-NT was from C.E.R. (Marloie, Belgium). All chemicals and solvents were of analytical grade. The C_{18} Bond Elut columns were purchased from Analytichem International (Interlaboratoire, Brussels, Belgium). The reagents for de-

rivatization, methylsilyltrifluoroacetamide (MSTFA), trimethylsilylimidazole (TMSI) and dithioerythritol (DTE), were from Macherey-Nagel (Düren, Germany), Sigma Chemie (Germany) and Aldrich (Brussels, Belgium), respectively.

Processing of urine and RIA procedure

These have been described previously [4]. Briefly, glucurono- and sulpho-conjugates of 19-NT in urine were hydrolysed enzymically, and cleaned-up on reversed-phase C_{18} Bond Elut columns (100 mg). The organic phase was evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in RIA buffer [0.02 *M* phosphate (pH 7.4)–0.5‰ gelatin].

A 100-μl volume of tritiated nortestosterone (*ca.* 10 000 dpm) in phosphate–gelatin buffer and 100 μl of diluted aniserum (1:21 000) were added to the 300 μl of sample extract solution. A standard curve was established using tubes containing increasing amounts of 19-NT (0, 6.25, 12.5, 25, 50, 100, 200, 400, 800 pg) and 200 μl of phosphate–gelatin buffer, 100 μl of tritiated 19-NT and 100 μl of diluted antiserum under the conditions described for unknown samples. All tubes were incubated at 37°C for 30 min and overnight at 4°C. A 500-μl volume of dextran-coated charcoal suspension (5 g of Norit charcoal and 0.5 g of dextran per litre of phosphate–gelatin buffer) were added. Tubes were incubated at 4°C for 10 min. After centrifugation at 1500 *g* for 10 min, 500 μl of the supernatant were taken off and mixed with 4 ml of Beckman HP/b scintillation cocktail. The radioactivity was determined using a Beckman LS 5000 (Analys, Ghent, Belgium). The results were calculated from a calibration curve after logit–log transformation and linear regression analysis.

Gas chromatographic–mass spectrometry analysis

For identification of 19-NT and its metabolites, urine samples were hydrolysed enzymically, cleaned-up on reversed-phase C_{18} Bond Elut columns (500 mg), spiked with 100 ng of methyltestosterone (internal standard for GC), evaporated to dryness, and derivatized by subsequent treatment with 50 μl of MSTFA (1000 μl), TMSI (2 μl) and DTE (2 mg) (unpublished procedure). GC–MS was performed under the following conditions: 70 eV; source temperature, 250°C; trap current, 100 μA. The equipment consisted of a HP 5790 gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) and a VG 7070 EQ spectrometer (VG Analytical, Manchester, U.K.).

RESULTS AND DISCUSSION

19-NT concentrations (ng/ml) measured by RIA are given in Figures 1, 4 and 6. A sample of blank urine taken from a pool of urine samples from untreated cattle that had been maintained under strictly controlled conditions, was measured in each assay. When the level of 19-NT in a sample was higher than the concentration in the blank urine + 2 ng/ml, we analysed the sample by GC–MS to confirm the possible endogenous presence of 19-NT.

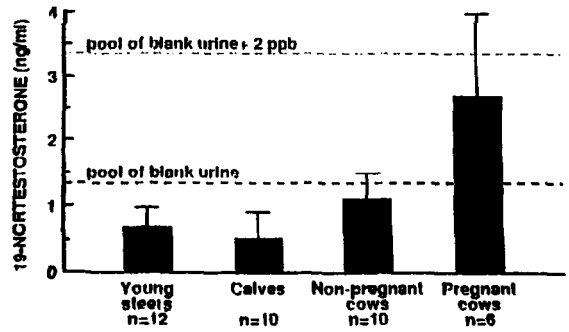

Fig. 1. 19-Nortestosterone levels (ng/ml) measured by RIA in cattle (error bar = 1 S.D.)

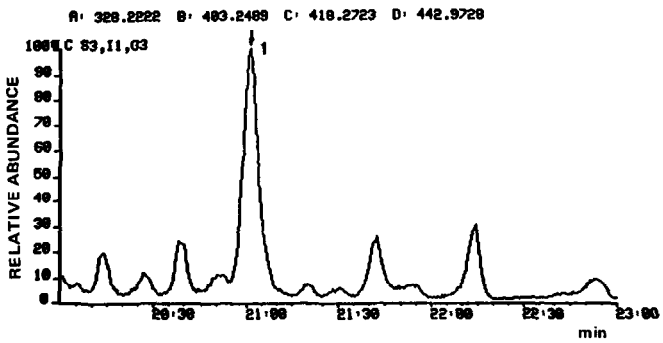

Fig. 2. Detection of 19-nortestosterone in urine of pregnant cows: confirmation of the presence of 17β-19-nortestosterone (m/z 418) with a retention time of 21 min (1).

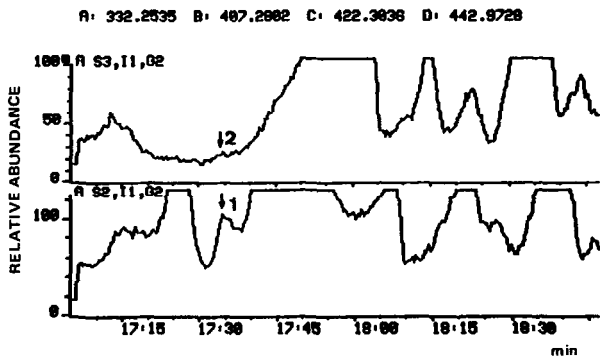

Fig. 3. Detection of a metabolite of 19-nortestosterone in urine of non-pregnant and pregnant cows: confirmation of the presence of oestrane-diol (m/z 332), with a retention time of 17 min 36 s, in the urine of a pregnant cow (1) but not in the urine of a non-pregnant cow (2).

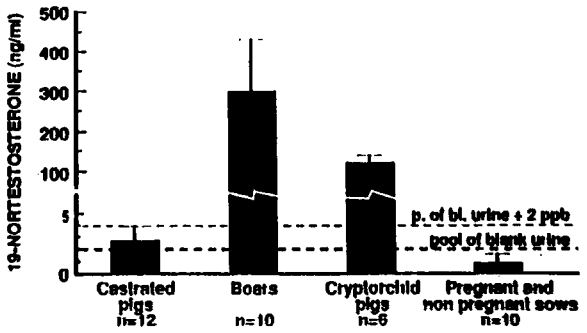

Fig. 4. 19-Nortestosterone levels (ng/ml) measured by RIA in pigs (error bar = 1 S.D.)

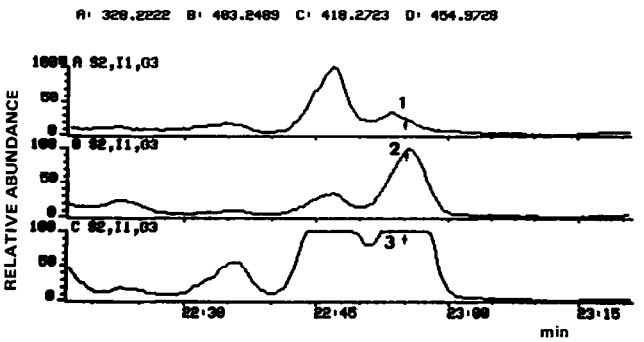

Fig. 5. Detection of 19-nortestosterone in boars: confirmation of the presence of 17β-19-nortestosterone, based on three ions (m/z 418, 403 and 328) with a retention time of 22 min 55 s (3,2 and 1).

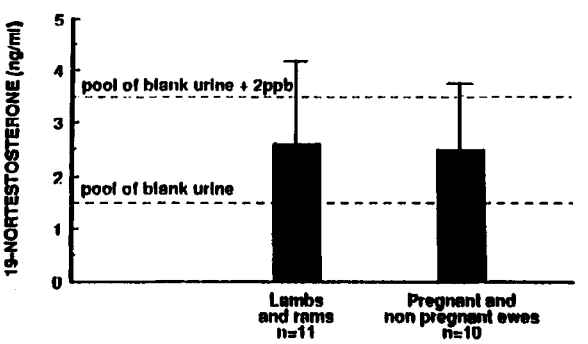

Fig. 6. 19-Nortestosterone levels (ng/ml) measured by RIA in sheep (error bar = 1 S.D.)

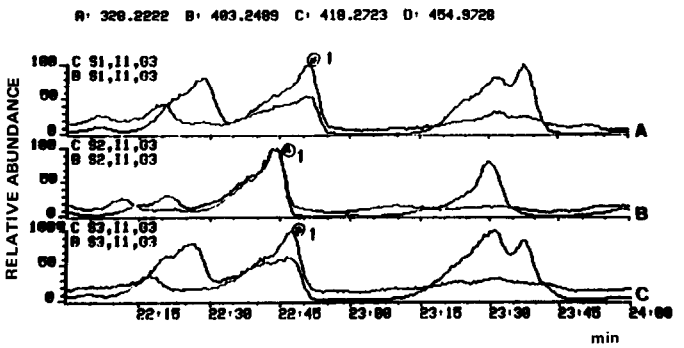

Fig. 7. Gas chromatograms of urine extracts from lambs and rams: a major peak (1) was detected in three samples (A, B and C) based on two ions characteristic for 17β-19-nortestosterone (*m/z* 418 and 403) with a retention time of 22 min 45 s which differs from that of authentic 17β-19-nortestosterone with a retention time of 22 min 55 s.

In cattle (Fig. 1), the level in urine from pregnant cows exceeds this limit of 2 ng/ml. Fig. 2 shows the gas chromatogram based on *m/z* 418 in a urine extract from a pregnant cow: 17β-19-NT was present with a retention time of 21 min. Oestrane-diol was also detected in these samples, but not in those from non-pregnant cows (Fig. 3), with a retention time of 17 min 36 s based on *m/z* 332.

The concentrations of 19-NT measured by RIA in urine from pigs (Fig. 4) are very high in boars and cryptorchid pigs. In castrated pigs, some levels near to the limit of 2 ppb were observed. The gas chromatogram of a boar urine extract shown in Fig. 5 confirms the endogenous presence of 17β-19-NT, with a retention time or 22 min 55 s, based on three ions (*m/z* 328, 403 and 418). The presence of the steroid was not confirmed in the castrates.

Fig. 6 summarizes the concentrations measured in urine from sheep. High levels are registered in samples from males as well as females. Nevertheless, 17β-19-NT was not detected in lambs' urine or ewes' urine (Figs. 7–9). Fig. 7 shows

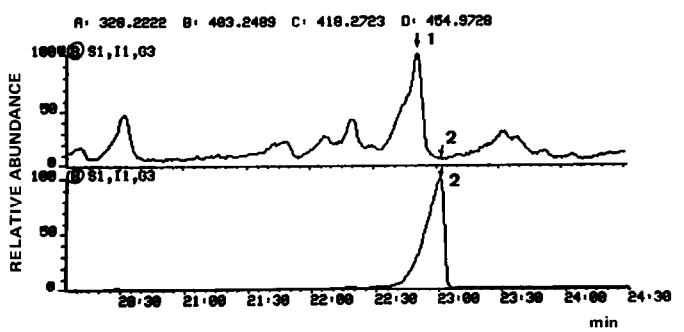

Fig. 8. Gas chromatograms of one of the urine samples analysed in Fig. 7: a sample of urine and the same sample spiked with 19-nortestosterone were studied by GC–MS. The major peak (1) with a retention time of 22 min 45 s does not correspond to the peak of 17β-19-nortestosterone with a retention time of 22 min 55 s (2).

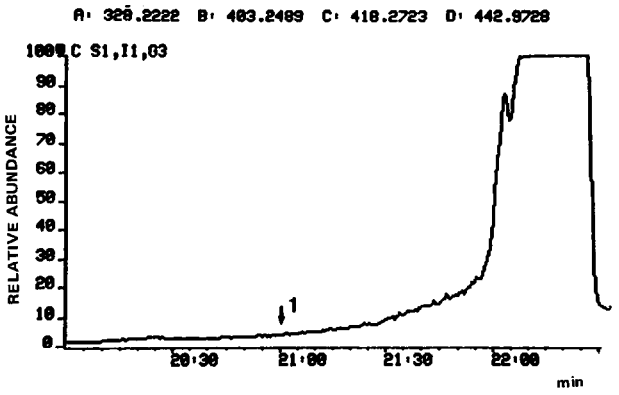

Fig. 9. Gas chromatogram of a urine extract from a pregnant ewe: 17β-19-nortestosterone (m/z 418) with a retention time of 21 min was not detected (1).

gas chromatograms of three urine extracts from male sheep, showing the presence of a major peak with a retention time of 22 min 45 s. 17β-19-NT has a retention time of 22 min 55 s. Spiking of one of the samples with 19-NT shows (Fig. 8) that this major peak does *not* correspond to the peak of 17β-19-NT.

The results of this study clearly demonstrate that the control strategy applied in the monitoring of the illegal use of 19-NT in meat-producing animals remains reliable. In cattle, urine samples from males and non-pregnant females can be analysed for the control of the illegal use of 19-NT. In pigs, pregnant and non-pregnant sows' urine can be monitored for control. However, the possible presence of endogenous 19-NT in urine samples from boars and cryptorchid pigs impedes the control of the use of 19-NT. This study reports for the first time the high levels of 19-NT (above the limit of 2 ppb) measured by RIA in urine samples from pregnant cows.

Since then, other laboratories have observed the same results. However, further investigations have demonstrated that difficulties are encountered in the GC–MS confirmation of the presence of this substance when 17α-estradiol is present in high concentrations. Indeed, the molecular ions of the enol-TMS derivatives of 17β-19-NT and 17α-estradiol are very close in mass (m/z 416 for 17α-estradiol and m/z 418 for 17β-19-NT) and in retention time. Consequently, if the 17α-estradiol level is much higher than that of 17β-19-NT, as is sometimes the case in urine samples from pregnant cows, these substances could be confused.

False-positive results are registered, by RIA, in urine samples from castrated pigs and sheep. The origin of the cross-reactions responsible for the high concentrations measured by RIA should be investigated.

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