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

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For the first time in the field of steroid residues in humans, demonstration of 19-norandrosterone (19-NA: 3α-hydroxy-5α-estran-17-one) and 19-noretiocholanolone (19-NE: 3α-hydroxy-5β-estran-17-one) excretion in urine subsequent to boar consumption is reported. Three male volunteers agreed to consume 310 g of tissues from the edible parts (meat, liver, heart and kidney) of a boar. The three individuals delivered urine samples before and during 24 h after meal intake. After deconjugation of phase II metabolites, purification and specific derivatisation of target metabolites, the urinary extracts were analysed by mass spectrometry. Identification was carried out using measurements obtained by gas chromatography/high resolution mass spectrometry (GC/HRMS) (R = 7000) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) (positive electrospray ionisation (ESI+)). Quantification was realised using a quadrupole mass filter. 19-NA and 19-NE concentrations in urine reached 3.1 to 7.5 µg/L nearby 10 hours after boar tissue consumption. Levels returned to endogenous values 24 hours after. These two steroids are usually exploited to confirm the exogenous administration of 19-nortestosterone (19-NT: 17β-hydroxyestr-4-en-3-one), especially in the antidoping field. We have thus proved that eating tissues of non-castrated male pork (in which 17β-nandrolone is present) might induce some false accusations of the abuse of nandrolone in antidoping. Copyright © 2000 John Wiley & Sons, Ltd.

17β-Nortestosterone, also called 17β-nandrolone (19-NT), one of the most powerful anabolic steroids, has been widely used in sport to improve muscular strength and performance and in meat producing animals to accelerate weight gain and to improve feeding efficiency. Synthesised by Birch[1] and by Wilds and Nelson[2,3] in the early 1950s, its metabolism was rapidly studied in man,[4] in horse[5] and in bovine.[6] This substance is banned in sport and in horseracing, as well as in feedlot production inside the European Union (EU). The control is generally based on the urine analysis of 19-NT metabolites, the parent compound being extensively altered. For instance, in man the two target metabolites which are considered to constitute irrefutable proof of 19-NT utilisation are 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE). However, since 1999, evidence for endogenous production of these metabolites has been demonstrated by Le Bizec et al.[7,8] and Dehennin et al.[9] Other works have been published during the last 15 years on other species, especially in the demonstration of endogenous existence of 19-nortestosterone metabolite forms. In 1984, the first findings were connected to stallions,[10-12] pregnant mares[13,14] and women.[13,15] In 1988, 17β-nandrolone was found in intact boar urine as well as in edible tissues.[16-19] Later, urine samples from pregnant cows, newborn calves[20-24] and finally pregnant ewes[25] were investigated and were proved to contain epinandrolone. All these data suggested to us that the consumption by humans of edible tissues from such animals should be equivalent to the oral administration of phase I or phase II nandrolone metabolites. Starting from the fact that pigs are the basis of a considerable number of delicacies, and nandrolone phase II metabolites in particular are present at high levels in their tissues, we focused our attention on the consequences of consumption of intact boar edible tissues on steroid urinary profiles in man. We especially tried to demonstrate whether eating liver, kidney or muscle (which contains ppb (µg/kg) levels of 19-NT) was compatible with the production of detectable concentrations of 19-norandrosterone and 19-noretiocholanolone in human urine. Then, having answered this in the affirmative, we planned to quantify metabolite urinary concentrations in order to check whether residual levels were below or above the decision limit fixed by International Olympic Committee (IOC) laboratories, in order to evaluate the risk of false accusations of the abuse of nandrolone associated with boar consumption.

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EXPERIMENTAL

Animal and meal preparation

A Piétrain double extra boar was slaughtered at 20.5 months. Its weight was 157.8 kg. A meal was prepared by a restaurant chef from boar kidneys, heart, meat and liver (roughly 25% of each component) by roasting and frying. Additional ingredients were vegetables, potatoes and a light mustard sauce made from the frying juice.

Test persons

Three healthy male volunteers agreed to consume the boar meal (Dirk, 24 years old, 192 cm, 91 kg, Kurt, 29 years old, 175 cm, 76 kg, and Luc 59 years old, 177 cm, 83 kg). The total amount consumed per person was 310 g of the prepared meat, equivalent to 375 g raw material (ca 90 g of kidney, liver, heart and meat). The three individuals delivered urine samples before the meal and up to 9 samples during the 24 hours after the consumption of boar meat. Urine samples were kept frozen below −18 °C until analysis. The experiment was conducted under supervision of a bailiff.

Reagents and chemicals

Most of the reagents and solvents were of analytical grade quality and provided by Merck (Darmstadt, Germany) and Solvants Documentation Synthesis (SDS, Peypin, France). The derivatisation reagents N-methyl-N-(trimethylsilyl)tri-fluoroacetamide (MSTFA) and trimethyliodosilane (TMIS) were purchased from Fluka (Buchs, Switzerland). Dithiothreitol (DTT) was from Aldrich (Milwaukee, WI, USA). Boehringer (St. Quentin Fallavier, France) provided β-glucuronidase (*Escherichia coli*).

Mass spectrometry and coupled chromatography

The quadrupole mass filter used was a HP 5970 instrument coupled to a 5890 gas chromatograph, both from Hewlett Packard (Palo Alto, CA, USA). The magnetic mass spectrometer was a reverse geometry double focusing instrument SX102A (Jeol, Tokyo, Japan) coupled to a HP-5890 gas chromatograph. The LC/MS/MS system was a triple quadrupole (QuattroLC®, Micromass, Manchester, UK) coupled to a HPLC system (Alliance 2690, Waters, Milford, MA, USA) via an electrospray interface (Z spray®, Micromass). Nitrogen was used as nebulisation and desolvation gas, at flow rates of 80 and 550 L/h. The potential applied on the capillary and on the cone were 4.0 kV and 20 V, respectively. MS/MS measurements were performed using argon as collision gas at a pressure of 4×10^{-4} mbar, the collision energy being 3 V. Reversed phase liquid chromatography was realised on octadecyl grafted silica. Flow rate was 220 µL/min, and the injected volume was 10 µL. The mobile phase used was methanol/water/acetic acid (80:20:0.2; v/v/v).

Sample preparation

Urine samples (10 µL) were hydrolysed (1 h, 52 °C, phosphate buffer pH 6.5) with 80 µL of β-glucuronidase from *E. coli*. The sample was then applied onto a C18 SPE column conditioned with methanol and water. Washing with water and hexane preceded analyte elution with 5 mL of methanol/ethyl acetate (30:70, v/v). A liquid-liquid clean up was performed twice with 2 mL of 1M sodium hydroxide.

After evaporation, hexane/ethyl acetate (85:15, v/v) was used to redissolve the dry residue before application onto a silica SPE column. Hexane/ethyl acetate (85:15, v/v) eluted the more non-polar interferences; 13 mL of a solvent of more eluotropic composition (60:40, v/v) achieved elution of the target steroids. After evaporation, the dry residue was redissolved in 30 µL of methanol/water (40:60, v/v). Final purification was achieved by semi-preparative HPLC based on a C18 stationary phase (15 × 0.2 cm i.d., 5 µm particle size) (Zorbax, Interchim, France) and on a methanol/water mobile phase (gradient from 40:60 to 10:90 (v/v) in 15 min followed by an isocratic period of 5 min). For LC/MS/MS measurement, the fraction containing target analytes was directly injected. For GC/MS analysis, the collected HPLC fraction was evaporated and derivatised for 40 min at 60 °C in 15 µL of MSTFA/TMIS/DTT 1000:5:5 (v/v/m).

RESULTS AND DISCUSSION

Detection of 19-NA and 19-NE

Urine samples collected before and after consumption of boar meat were initially analysed by GC with a low resolution mass spectrometer (selected ion monitoring (SIM) acquisition). 19-Noretiocholanolone-d_3 and methyl-testosterone-d_3 were added as internal and external standards, respectively, in order to certify the efficiency of the extraction-purification process and to prove the performance of the derivatisation step. This procedure provided analyte concentration and extraction yield values. Figure 1 shows typical ion chromatograms of m/z 405 and 408 of different urine samples collected before and after the boar meal consumption. The internal standard, 19-NE-d_3, was detected at 12.72 min (m/z 408); signals corresponding to 19-NA and 19-NE occurred at 12.39 and 12.73 min, respectively (m/z 420, 405, 315, 225). Some traces of these two steroid analytes are present in low concentration in the reference urine (H_0), which correspond to the endogenous 19-NA and 19-NE (<0.01 ppb) levels. One hundred and five minutes after meal intake, levels of the two metabolites slightly increased to reach 0.02 and 0.01 ng/mL for 19-NA and 19-NE, respectively. The concentration progressed drastically three hours and fifty minutes after boar ingestion, to attain 3.2 and 0.8 ppb, respectively. Twenty-four hours after meal consumption, the levels returned to the endogenous values, i.e. around 0.1 ppb.

Identification of 19-NA and 19-NE

Several analytical approaches have been used to confirm the analyte identity.

Low resolution GC/MS. The first was performed by GC/MS measurement on a low resolution quadrupole mass spectrometer in SIM mode (Fig. 2). Relative retention time of the analyte must correspond to that of the calibration standard at a tolerance of ±0.5% to be considered as still suspect. Four ion intensity ratios of each of the two metabolites were then considered and compared with the corresponding standard analytes. The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion, must correspond to those of the standard analyte in spiked sample with a tolerance of ±10% to be considered as unambiguously identified. The two SIM mass spectra of the suspected 19-norandrosterone (MS A) and that of 19-NA

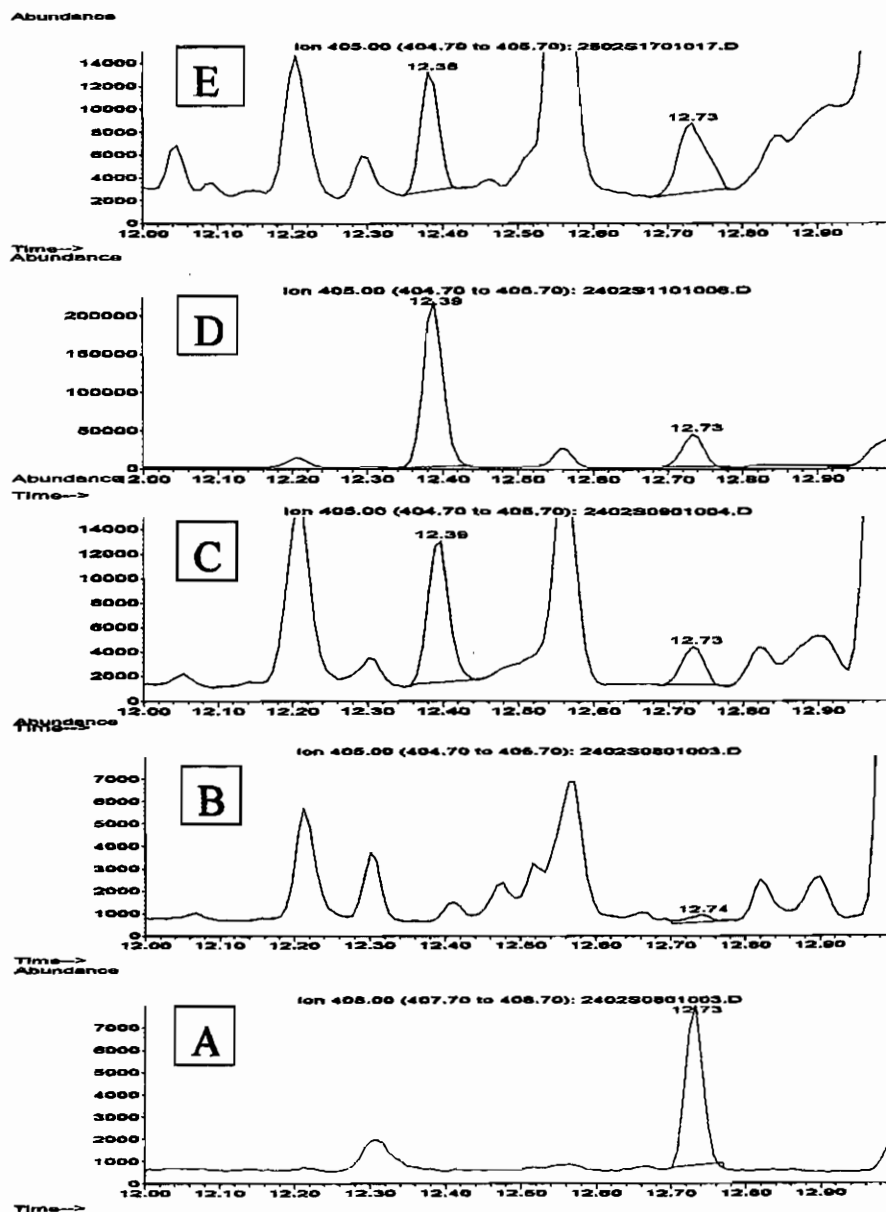**Figure 1.** GC/MS measurement of DIRK urine samples collected just before consumption of the boar meal (B: H_0) and after (C: H_0 + 1h45; D: H_0 + 3h50; E: H_0 + 24h05). m/z 408 ion chromatogram (A) corresponded 19-NE-d_3 (12.73 min), m/z 405 ion chromatograms (B to E) displayed 19-NA (12.39) and 19-NE (12.73) min signals.

standard (MS B) are shown in Fig. 2. They proved to be within the tolerance of ±10%.

High resolution GC/MS. The second confirmation was performed by GC/MS measurement in SIM mode on a magnetic mass spectrometer (inverse geometry) at 7000 resolution (10% valley). Four exact masses were monitored for the two nandrolone metabolites (m/z 420.2880, 405.2645, 315.2144, 225.1643) and one for 19-NE-d_3 (m/z 408.2833). The results for the urine sample corresponding to 5h25min after the boar meal intake (LUC) are shown in Fig. 3. The four ion chromatograms are free of interferences at the expected retention time of steroid metabolites. 19-NA (13.98 min) and 19-NE (14.59 min) were estimated to be 2.7 and 0.6 ppb, respectively. A further confirmation was performed on the same urine sample by acquiring signals of the theoretical exact masses of the 19-NA and 19-NE

molecular ion (m/z 420.2880) and mass spectrum base peak (m/z 405.2645), together with the ions at m/z ± 10 mmu (millimass unit) (Fig. 4). The abundances of the resulting signals were compared. Table 1 summarises the results of this experiment. Areas corresponding to each ion signal were systematically more important for the theoretical elemental composition (420.2880 and 405.2645) compared with neighbouring m/z values. The internal standard, deuterated 19-noretiocholanolone, validated this approach; its theoretical exact mass (m/z 408.2833) being the most intense measured signal. We thus reinforced the identity confirmation of the two steroid metabolites by confirming each ion's elemental composition through their exact masses.

LC/MS/MS. The third confirmation was realised by LC/MS/MS measurement on a triple quadrupole mass filter
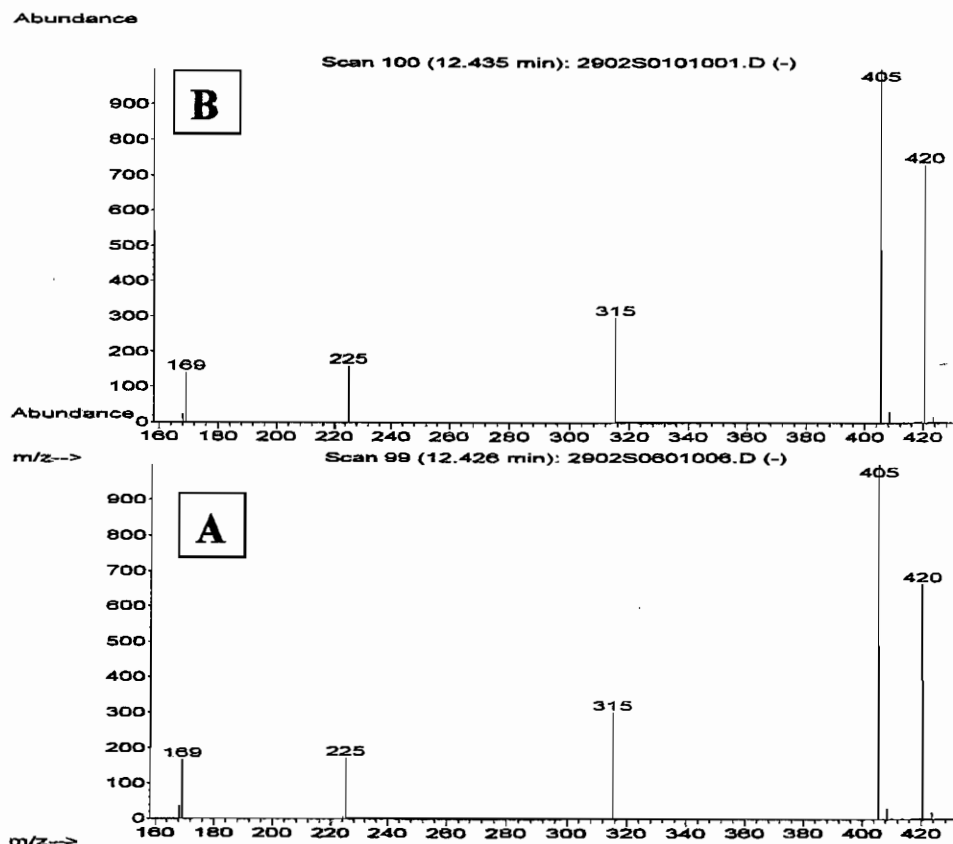
Abundance

Figure 2. SIM mass spectrum of a DIRK urine sample collected 8h35min after consumption of the boar meal (MS A = DIRK: H_0 + 8h35min) and a spiked urine sample (MS B). Retention times (12.426 and 12.435 min) and ion ratios were in the tolerance range.

using positive electrospray ionisation. The transitions monitored were $MH^+ > (MH-H_2O)^+$ and $MH^+ > (MH-2H_2O)^+$, i.e. $277 > 259$ and $277 > 241$ for 19-NA and 19-NE. Ion chromatograms corresponding to a spiked blank and to a urine sample collected 5h25min after the boar meal intake are shown in Fig. 5. 19-NE and 19-NA MRM signals are visible at 11.40 and 11.72 min, respectively. Relative intensities of the two transitions are different for the two metabolites which differ in the α/β position of the hydrogen atom on C5. For instance, 19-noretiocholanolone demonstrated a more important propensity to lose two H_2O molecules compared with 19-norandrosterone. In any event, transition intensities in this urine sample (DIRK H_0 + 5h25min) were in agreement with those for the corresponding reference spiked blank sample.

Identification conclusion. The combination of low and high resolution GC/MS with LC/MS/MS (ESI+) permitted the unambiguous identification of 19-norandrosterone and 19-noretiocholanolone in the urine samples of the three individuals after intake of boar edible tissues. This step was absolutely necessary before any quantification.

Quantification of 19-NA and 19-NE

After a preliminary estimation of the steroid analytes, a calibration curve was performed taken into account these initial indications. For 19-noretiocholanolone, one calibration curve was acquired between 0.1 and 1.3 ng/mL. For 19-norandrosterone, two calibration curves were obtained in

order to be as precise as possible in the two extreme concentration regions. One covered 0.1 to 5.0 ppb, whereas the second covered from 2.5 to 10.0 ppb. The two calibration curves corresponding to the low concentration sections are shown in Fig. 6. The response linearity was judged acceptable, the correlation coefficients being 0.9959 and 0.9982 for 19-NA and 19-NE, respectively. A summary of the results obtained for each of the three individuals over 24 hours is shown in Fig. 7 and Table 2. In the three individuals, almost the same urinary excretion profiles were observed. Indeed, 24 hours after boar intake the residue levels returned to their original concentrations, i.e. their endogenous values. The maximal concentrations were observed between 3 and 17h after consumption. 19-Norandrosterone concentrations above the 2 ppb decision limit applied by IOC laboratories were found in several urine samples. For DIRK, five urine samples (between 3h50 and 16h15), for KURT, one urine sample (9h40) and for LUC, three urine samples (between 5h25 and 11h45) were concerned. For the three individuals, the maximal values reached for 19-NA were 7.5, 3.7 and 3.1 µg/L. For 19-NE, highest concentrations were 1.0, 0.5 and 1.2 µg/L. Moreover, one can observe that 19-NE concentrations decreased more slowly in comparison with those of 19-NA, so that residual levels remained above 0.1 ppb after 24 hours in two individuals. The monitoring of the 19-NA/19-NE ratio over time, especially for urine samples above 1 ppb for 19-NA, yielded mean values between 5 and 6. Intra-individual ratios (R) remained to a certain extent homogenous, varying between R and 2R. The ratios fluctuated between 4.1–7.5
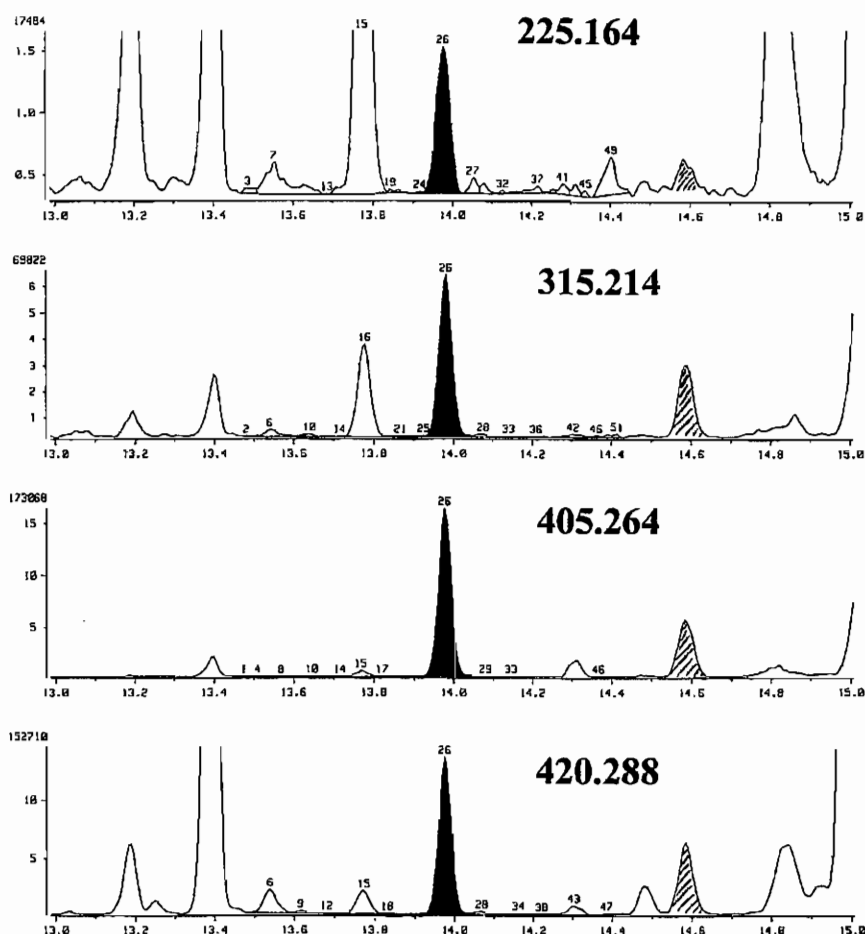
Figure 3. HR-SIM (R = 10000) ion chromatogram corresponding to a urine sample collected 5h25min after boar meal intake (LUC: H_0 + 5h25). Ion chromatogram signals (m/z 420.2880/405.2645/315.2144/225.1643) permitted attribution of the peaks at 13.98 and 14.59 min to 19-NA and 19-NE.

for DIRK, 5.4–10.4 for KURT and 2.5–4.2 for LUC. These values are not significantly different from that observed after exogenous administration of 19-nortestosterone to human.[26]

Table 1. Verification of the elemental composition of the detected steroid metabolites. The elemental composition was recorded during the same acquisition (R = 7000 at 10% valley, RT: retention time)

Molecule	Theoretical m/z	Recorded m/z	RT (min)	Area
		420.2780		53.7159
	420.2880	420.2880		57.6620
19-Norandrosterone		420.2980	14.02	40.9141
		405.2545		38.5637
	405.2645	405.2645		46.4109
		405.2745		34.0001
		420.2780		12.2769
	420.2880	420.2880		13.9663
19-Noretiocholanolone		420.2980	14.63	10.3392
		405.2545		7.4686
	405.2645	405.2645		8.8766
		405.2745		6.4055
		408.2733		4.6489
19-Noretiocholanolone-d$_3$	408.2833	408.2833	14.60	5.4435
		408.2933		4.8795

CONCLUSIONS

The work presented here has demonstrated unambiguously the incidence of a boar meal intake on possible false accusations of the abuse of nandrolone in antidoping control. We established that the consumption of male pig edible tissues was able to generate, within a 24 h period, several μg/L (ppb) of 19-norandrosterone and 19-noretiocholanolone in urine. These two steroids, generally used to prove the illegal use of 19-nortestosterone, were still above 1 ppb, 15 hours after boar ingestion, in the three individuals tested. The IOC decision limit, i.e. 2 ppb, was exceeded during several hours, no matter which individual was studied. Maximum concentrations were variable depending on the subject, reaching at most 3.1 to 7.5 ppb. Ratios between metabolites appeared not significantly different from a classical nandrolone administration, in any case in favour of 19-norandosterone (NA/NE in between 2.5 and 10 with most values between 5 and 6). A certain number of complementary studies must still be carried out. For instance, we are investigating the relative proportions of phase II metabolites, the measured concentrations presented in this study being relative to free plus glucuronic metabolites. One unknown in the study is, for the time being, the origin of excreted 19-NA and 19-NE; a study has been initiated to identify and quantify 17β-nandrolone forms
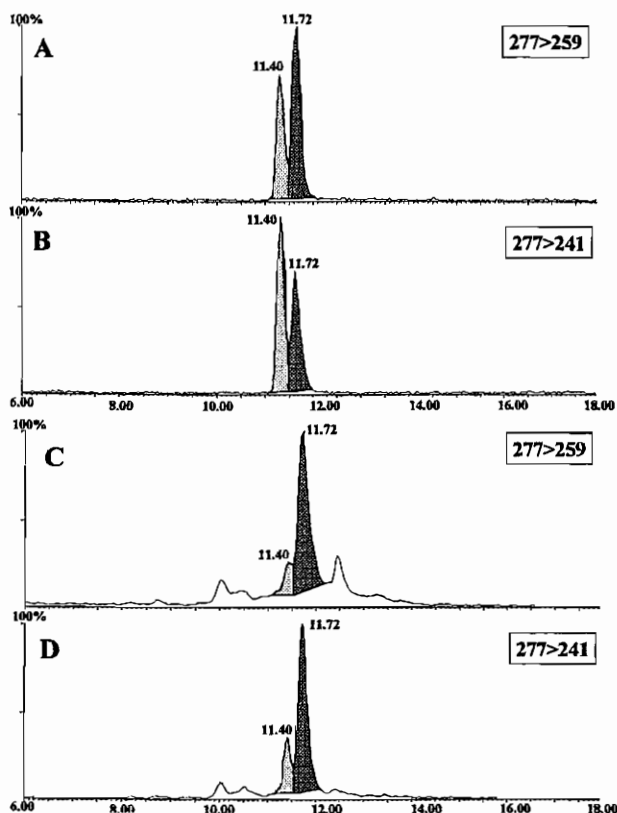
Figure 5. LC/MS/MS ion chromatograms (positive electrospray, MRM acquisition) corresponding to a spiked urine sample (A and B) and to a urine sample collected 5h25min after boar meal intake (C and D). Acquired transitions were 277 > 259 and 277 > 241.
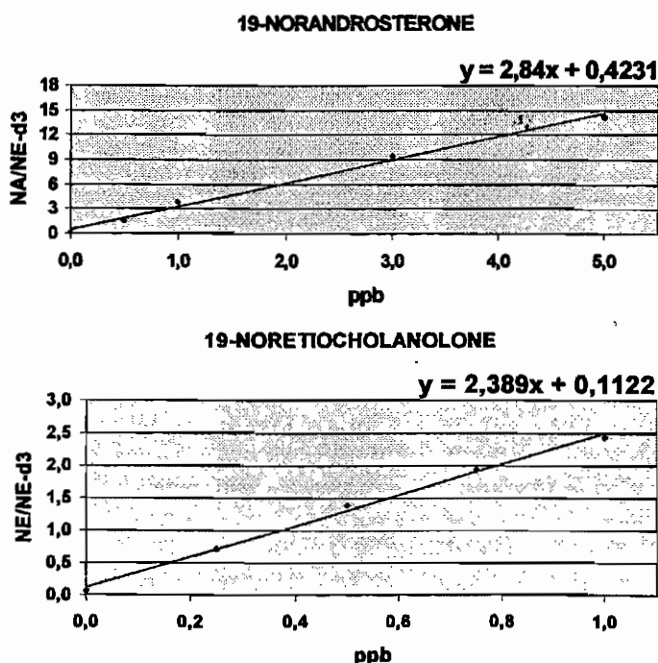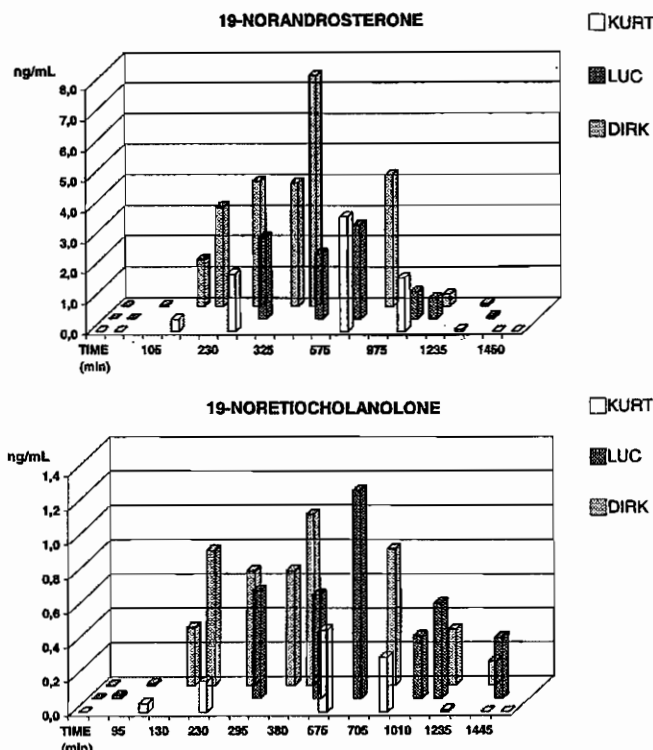

Figure 7. Kinetic elimination of 19-NA and 19-NE in urine after boar meal intake by the three volunteers (KURT: blank, LUC: black, DIRK: grey). Y-axis corresponds to the concentration of each metabolite in ng/mL. X-axis expresses the time in minutes after meal consumption.

Figure 6. Calibration curves for 19-norandrosterone and 19-noretio-cholanolone. Y-axis corresponds to the height ratio between m/z 405 (19-NA and 19-NE mass spectrum base peak) and m/z 408 (19-NE-d$_3$). X-axis expresses concentration of 19-NA and 19-NE in ng/mL (ppb). Equation is indicated for each analyte.

(free, glucuronic or sulfate conjugates) responsible for such excretion. Existence of other precursors in entire male pig edible tissues is also under consideration. Finally, a method based on gas chromatography/carbon isotope ratio mass spectrometry (GC/C-IRMS) is under development for evaluation as a means to distinguish food contamination from illegal use of nandrolone.

Acknowledgements

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