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Excretion of norsteroids' phase II metabolites of different origin in human

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ABSTRACT

The urinary phase II metabolites of norsteroids, 19-norandrosterone, 19-noretiocholanolone and 19-norepiandrosterone glucuronide and sulphate, were analyzed in samples collected during the pregnancy, following the administration of norsteroids or the consumption of edible parts of non-castrated pig and in athletes' samples in which they were found during routine controls. The level of the sulfo- and glucuroconjugated metabolites was precisely determined by GC/HRMS, after selective hydrolysis. The goal was to evaluate whether the fine analysis of the norsteroid conjugates produced and excreted in different conditions would show a pattern that could be linked to their origin. The delta ^{13}C values of the metabolites formed following the ingestion of edible parts of non-castrated pig were measured by isotope ratio mass spectrometry. Our results indicated that it is not possible to determine the origin of the urinary metabolites based upon the sole evaluation of the different metabolites and conjugates. The GC/C/IRMS is the only method permitting to distinguish between the exogenous and endogenous origin of the metabolites.

1. Introduction

The administration of pharmaceutical preparations of *nandrolone* (19-nortestosterone, 17β-hydroxyestr-4-en-3-one), a known anabolic steroid, and of other norsteroids sold as "pro-hormones", lead mainly to the excretion of 19-norandrosterone (19-NA; 3α-hydroxy-5α-androstan-17-one), 19-noretiocholanolone (19-NE; 3α-hydroxy-5β-androstan-17-one) and 19-norepiandrosterone (19-NEA; 3β-hydroxy-5α-androstan-17-one). The latter possessing a 3β-hydroxyl group is almost exclusively sulfoconjugated, while the first two are predominantly glucuroconjugated. 19-norandrosterone is generally excreted predominantly over the 5β-isomer but inversed proportions were reported at the end of the excretion period or when Δ^5-isomers of norsteroids were taken [1–7]. Nortestosterone and the precursors, norandrostenedione and norandrostenediol are listed as prohibited substances by the World Anti-Doping Agency and the presence of 19-NA in an amount greater than 2 ng/mL (adjusted to a specific gravity of 1.020) in athletes' samples shall be reported as an adverse finding [8].

The presence of 19-NA in human urine samples was reviewed lately [9]. Excreted in very low amounts in human urine samples, endogenous 19-NA is not detected by the

Abbreviations: 19-NA, 19-norandrosterone (5α-estran-3α-ol-17-one); 19-NE, 19-noretiocholanolone (5β-estran-3α-ol-17-one); 19-NEA, 19-norepiandrosterone (5α-estran-3β-ol-17-one); 19-NT, 19-nortestosterone (Estr-4-en-17β-ol-3-one); Nordione, 19-norandrost-4-en-3,17-dione (Estr-4-en-3,17-dione); IRMS, isotope ratio mass spectrometry; HRMS, high-resolution mass spectrometry.

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methods routinely employed in drug testing laboratories; the threshold being fixed at 2 ng/mL, the required performance level is 1 ng/mL [10]. Higher levels reaching up to 15–20 ng/mL are measured in specimens collected during pregnancy [11]. In the specimens of some sportsmen and male volunteers, 19-NA was found at levels varying for example around 0.01–0.32 (mean 0.08 ng/mL) [12] or at 0.05–0.6 ng/mL [13], thus well below the limit for reporting positive results. It appears that high-intensity exercises do not influence the excretion of 19-norsteroids administered in trained athletes and again, very low levels ranging from undetectable to a maximum of 0.25 ng/mL were measured (mean value of 0.048 ± 0.050 ng/mL) [14]. The physiological levels of 19-NA measured in samples collected from females are also lower than 1 ng/mL [15–17], maximum value of 0.8 ng/mL having been recorded during the ovulation, correlating apparently with high levels of estrogens [18]. In tissues expressing the cytochrome P450 aromatase-complex, 19-nortestosterone and 19-norandrost-4-en-3,17-dione seem to be formed *in vitro* during the aromatization of androgens to oestrogens [19–23]. In humans, norsteroids were detected in ovarian follicular fluids and in the placenta, one group proposing a model according to which norsteroids would be formed in a relation of 8:72 to estradiol during the aromatization process, while it was reported that the excretion of 19-NA could derive from gonadal stimulation, having observed that basal levels were being increased to around 0.4 ng/mL further to the administration of hCG [24–26].

In the past years, numerous studies confirmed that products sold as nutritional or sport supplements were not properly labeled and contained steroids (related to testosterone or nortestosterone); some athletes who tested positive were able to link the test result to a mislabeled commercial product [27–33].

More recently, the formation of trace amounts of norsteroids metabolites by *in situ* 19-demethylation of etiocholanolone (3α-hydroxy-5β-androstan-17-one) and androsterone (3α-hydroxy-5α-androstan-17-one) was observed in athlete's samples upon incubation. The reaction being more favorable in 5β-steroids, 19-NA and 19-NE appear in ratios lower than that of their respective urinary precursors, androsterone and etiocholanolone [34]. The criteria for reporting adverse findings were reviewed to include verification steps in the relatively rare samples showing characteristic criteria of unstable urines [35]. The origin of 19-NA present in amounts as low as 2–3 ng/mL can now be determined by GC/C/IRMS in a relatively low volume of urine sample making it suitable for confirmation of athlete's samples [36,37].

19-norsteroids, 17α- or β-isomers, are produced naturally in some animal species such as the stallion, the non-castrated pig, female in gestation and were found in the newly born calf [38–48]. A first report described the ingestion of a substantial amount of non-castrated pig meat (375 g) resulting in the excretion of 19-NA in amounts reaching 3–7.5 ppb in the following hours [49].

This work aims at studying the phase II metabolites originating physiologically during the pregnancy or from the ingestion of norsteroids from "hormonal supplement" of 19-norandros-4-en-3,17-dione or contained in edible parts of non-castrated pig and finally, in several athlete's samples which were found to be positive during routine doping controls. We have estimated by GC/HRMS the excreted levels of 19-NA, 19-NE and 19-NEA when possible, after selective hydrolysis of the glucuro- and sulfoconjugated metabolites. The delta ^{13}C values of the metabolites formed following the ingestion of edible parts of non-castrated pig were measured by isotope ratio mass spectrometry.

2. Experimental

2.1. Reagents, solvents, chemicals and standards

All chemicals, salts and buffers were of analytical grade and obtained form either J.T. Baker (Phillipsburg, NJ, USA), Fisher Scientific (Montreal, QC, Canada), BDH (Montreal, QC, Canada) or Malinckrodt (Paris, KY, USA). β-glucuronidase from *E. coli* type IX-A lyophilized powder containing between 1.5 and 2×10^6 U/g was purchased from Sigma Chemical (St. Louis, MO, USA). The chemical derivatization agents, trimethyliodosilane (TMIS) and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased form Aldrich (Milwakee, WI, USA), triethylamine from Sigma Chemical and ethanethiol from Fisher (NJ, USA). The solvents were glass-distilled or HPLC grades and were purchased from Caledon (Montreal, QC, Canada). Deionized water was obtained by reverse osmosis and filtered over a Milli-Q water purification system (Millipore, Montreal, QC, Canada). Nitrogen, grade zero, was obtained from Air Liquide Canada (Montreal, QC, Canada). Commercial capsules of 19-norandrost-4-en-3,17-one (*Nordione*) were purchased 8 years ago with Health Canada's authorization (number: 8572.090.98) from a distributor of "sport nutritional supplements" chosen randomly on the Internet, Price's Power International (International Nutrition and Export, Newport News, VA, USA). Standards of 5α-estran-3α-ol-17-one and 5β-estran-3α-ol-17-one were obtained from Radian International (Austin, TX, USA), certified 5α-estran-3α-ol-17-one and 5β-estran-3α-ol-17-one from Cerilliant (Austin, TX, USA), 5α-estran-3β-ol-17-one and 5α-estran-3α,17β-diol from Steraloids Inc. (Wilton, NH, USA).

2.2. Excretion studies

Excretion studies were conducted with the approval of the Ethical Committee of INRS in 2000; all urine samples were collected in sterile plastic containers and were kept frozen at–20 °C until analyzed. (A) Pregnancy: urine samples were collected once a week during the entire pregnancy of two women aged 30 (W30) and 34 (W34) respectively. (B) Administration of 19-norandrost-4-en-3,17-dione: one capsule of *Nordione* containing 100 mg of 19-norandrost-4-en-3,17-dione was administered to a healthy male volunteer (M26; 26-year-old). The content of the pill was verified by GC/MS. All the specimens were collected 24 h before and 5 days after the administration. (C) Athletes' urine samples containing norsteroids: ten athletes' urine samples analyzed in our laboratory and reported positive for the presence of norsteroids' metabolites during routine doping controls were selected for re-analysis. These samples were collected between 1993 and

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2000 during a period where clear adverse findings were due to the administration of *nandrolone* pharmaceutical preparations, were kept frozen since then. (D) Consumption of pig edible parts: selected edible parts of three 3-year-old uncastrated pigs and of three 4-month-old castrated pigs were provided by a local slaughterhouse (*Oviande* Inc., Montreal, QC, Canada) and were kept frozen. The *Institut du tourisme et d'hôtellerie du Québec* (ITHQ, Montreal, Canada) prepared on two different occasions, different meals each containing a total amount of 300 g (18 volunteers) or 100 g (3 volunteers) of cooked meat. In the first round, three different meals containing 300 g of either uncastrated or castrated pig edible parts were prepared: the first combined roasted kidney, liver and heart (100 g of each), the second one, liver and heart (150 g of each) while the third one contained only oven-baked meat (leg). The 18 volunteers who agreed to participate in the first "300 g" experiment (double-blind study) were healthy men and women aged between 22 and 53 and weighing 56–95 kg. Six different groups of three were made including at least one man and one woman in each group. One individual in each group had to exercise for a period of 1 h. On a second round of experiments, three other meals were prepared for 3 volunteers who had participated before, this time with 100 g of kidney, liver or meat of uncastrated pig only. All the urines samples were collected during 24 h before and 48 h after the meal. They were kept frozen until analyzed.

2.3. Analysis of samples

The urinary norsteroids were detected, identified and their concentration measured by GC/MS analysis in the selected ion monitoring mode (SIM) as their TMS-derivatives. A first screening of the samples was done on the combined free and glucuroconjugated fraction in order to identify and estimate the amount of the norsteroids present and to exclude an administration of other anabolic agents. For the final confirmation and quantification by GC/HRMS, glucuro- and sulfoconjugated steroids were extracted by selective hydrolysis. The four samples containing the highest amount of norsteroids derived from the consumption of uncastrated pig offal were analyzed by GC/C/IRMS.

2.4. Preparation of the urine samples

The volume of urine was adjusted in function of the specific gravity of the sample (ranging from 1.5 to 10.0 mL); aliquots were diluted with an equal volume of acetate buffer (pH 5.2, 2 M). After the addition of 125 ng of the internal standard, 5α-estran-3α,17β-diol, the steroids were isolated by solid phase extraction (Sep Pak plus tC_{18} cartridges (Waters, Canada). The glucuroconjugated steroids contained in the methanolic eluate were hydrolyzed after evaporation to dryness, with 1 mg of the enzymatic preparation in 1 mL phosphate buffer (pH 6.9) at 50 °C during 1 h. The samples were then treated as described previously to form TMS-ether, TMS-enol derivatives [50]. For the confirmation and quantification experiments, new aliquots were prepared (triplicates) adjusting the volume of urine in order to be within range of linearity of the method. Blank urine and quality control specimens were processed simultaneously. The glucuroconjugated steroids were isolated and hydrolyzed as described and extracted twice with hexane at pH 11 [51]. The solvolysis of the steroids was carried out on the residue at 50 °C for 1 h with 2 μL of sulphuric acid (4 M) in 1 mL of tetrahydrofuran as described previously [52] and the steroids then extracted twice with diethylether at pH 9. The TMS-ether, TMS-enol derivative mixtures were analyzed as such by GC/HRMS.

2.5. Preparation of urinary standards

For the calibration curves, authentic standards of 19-NA, 19-NE and 19-NEA were spiked in child urine in concentration ranging from 0.5 to 15.0 ng/mL and so were quality control samples containing 2.0, 4.0, 5.0 and 8.0 ng/mL of norsteroids. The limits of detection and quantification were established for the present experiments using urine samples spiked in amounts of 0.01–1.0 ng/mL.

2.6. GC/MS and GC/HRMS analysis

Verification of the identity of the steroids, first screening and estimation of the concentration of norsteroids was done by GC/MS using a HP 5973 MSD instrument linked to a HP 6890 gas chromatograph and an autosampler HP 7683 (Agilent Technologies) with a HP-5MS capillary column (5% phenyl polymethyl siloxane, 25 m × 0.20 mm inside diameter, 0.33 μm film thickness: Agilent Technologies). Typically, 1 μL of the TMS-derivatization mixture was injected in the splitless mode with the following instrument conditions: injector 270 °C, transfer line 310 °C with programming of the oven temperature starting at 100 °C (hold 1 min) increased at 20 °C/min to 220 °C, then to 278 °C at 4.4 °C/min to 320 °C at 10 °C/min (hold 5.6 min). 19-NA and 19-NE were detected in the SIM mode with the ions at *m/z* 420.3 (molecular ion), 405.3 and 315.2 and 422.3, 407.3 for the internal standard. The samples were grouped in batches of 20 including one blank urine and a control sample containing 19-NA and 19-NE in a concentration of 3.0 ng/mL (0.06 ng/μL injected). The absolute retention times and ion ratios had to correspond to those of the authentic standards within 5% and 10% respectively. The limit of detection was 0.3 ng/mL (S/N = 3).

Quantification was performed on a JEOL JMS-700 high-resolution mass spectrometer (JEOL, Tokyo, Japan) linked directly to a HP 6890 gas chromatograph and HP 7683 autosampler (Agilent Technologies) with the same capillary column and conditions of separation. Operating conditions were as follows: ionization current 300 μA, accelerating voltage 10 kV, electron multiplier 1 kV, and resolution 10,000 or 11,000. The ions were fixed at *m/z* 420.2879, 405.2645, 315.2144 for 19-NA, 19-NE and 19-NEA and at *m/z* 422.3036 and 407.2802 for the internal standard. The limit of detection of these experiments was 0.04 and 0.05 ng/mL respectively for 19-NA and 19-NE (S/N = 3) and the limit of quantification, 0.07 and 0.08 ng/mL respectively (S/N = 5) as estimated with a urine sample of a specific gravity of 1.012. The method was linear over the range of concentrations under study, i.e., from 0.5 to 15.0 ng/mL, coefficients of correlation for the calibration curves (linear, including origin) better than 0.995 and the QC values found within 15% of variation from the theoretical values. When specified, to allow comparison between sam-

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ples, the concentration was adjusted taking into account the specific gravity of the specimens as proposed by Donike et al. [53]: $[C]_{corrected\ 1.020} = [C]_{measured} \cdot \{(1.020 - 0.998)/(\text{specific gravity} - 0.998)\}$.

2.7. GC/C/IRMS

(a) The delta ^{13}C values were carried out at the Laboratory in Koln, Germany. *Sample Preparation*: the steroids were isolated from 10 mL of urine with 500 mg SPE cartridge (Chromabond C18, Macherey & Nagel, Düren, Germany). The methanol eluate was evaporated to dryness, the residue dissolved in 1 mL of sodium phosphate buffer (0.2 M, pH 7) and then washed with 5 mL *tert*-butyl methyl ether (TBME). After careful removal of the residual TBME, the glucuroconjugated steroids were hydrolyzed as described previously with β-glucuronidase from *E. coli* (50 °C/2 h). After the addition of 250 μL of a 20% aqueous solution of a 1:1 mixture of K_2CO_3:$KHCO_3$, the steroids were extracted with 5 mL of TBME. The residue was dissolved in 50 μL of methanol to which was added 1 μL of a methanolic solution of testosterone acetate (1 mg/mL) as the reference standard for the separation by HPLC. *Isolation of steroids by HPLC*: the steroids of interest were purified using an Agilent 1100 (Agilent, Böblingen, Germany) liquid chromatograph equipped with a variable wavelength detector and a LiChrospher RP-18 column (250 mm × 4 mm, particle size of 5 μm). The mobile phase was composed of a gradient of water and acetonitrile, starting at 30% CH_3CN increasing linearly to 45% in 17 min and then to 100% within another 5 min. The column flow was kept at 1.8 mL/min and compounds were detected at 192 nm. Three fractions were collected (LC-1–LC-3). Noretiocholanolone was recovered in the first (LC-1, 13.5–14.9 min), norandrosterone in the second (LC-2, 14.9–16.1 min) and etiocholanolone often associated with androsterone in the third fraction (LC-3, 16.1–18.3 min). The fractions were evaporated and the residue was dissolved in 10 μL of cyclohexane. The solution containing free steroids was analyzed as such.

Acetylated steroids: after removal of the solvent, the steroids were acetylated by the addition of 5 μL of pyridine, 150 μL of acetonitrile and 150 μL of acetic anhydride and heating at 80 °C for 2 h. The mixture was purified by HPLC under the same conditions under a gradient solvent of acetonitrile and water starting at 40% acetonitrile increasing to 100% in 15 min. Acetylated norandrosterone was recovered in the fraction collected between 10.4 and 11.3 min. The fraction was treated as described previously and analyzed as such. *GC/C/IRMS*: free underivatized steroids were analyzed on a HP 5890 gas chromatograph coupled to a Finnigan MAT delta-C (Finnigan MAT, Bremen, Germany) isotope ratio mass spectrometer. Acetylated compounds were analyzed on a HP 6890 gas chromatograph coupled to a Finnigan MAT delta-plus XP isotope ratio mass spectrometer. *Analysis of underivatized steroids*: the free steroids were injected on an Optima-δ 3 column (Macherey & Nagel, Düren, Germany; 17 m × 0.25 mm, film thickness: 0.25 μm). The injection port was kept at 300 °C and the injection was made in the splitless mode. Deactivated fused silica (SGE, 2 m length, 0.32 mm inner diameter) connected in front of the GC column by a pressfit connector served as retention gap. The temperature of the oven was programmed as follows: 60 °C (1.5 min), increased to 256 °C at 30 °C/min to 295 °C at 3 °C/min (kept for 4 min). Four reference gas pulses were set when in "straight mode" to calculate $^{13}C/^{12}C$ values. *Analysis of acetylated steroids*: the steroids were injected on an Optima-δ 6 column (30 m × 0.25 mm; film thickness: 0.25 μm) under the same conditions described above. The oven temperature was programmed as follows: 60 °C (1.5 min) to 280 °C at 40 °C/min and to 300 °C at a rate of 2 °C/min (final temperature kept for 5 min).

3. Results and discussion

3.1. Administration of a "dietary supplement" of 19-norandrost-4-en-3,17-dione

As shown in Fig. 1, 19-norandrostenedione is rapidly metabolized to 19-NA and 19-NE excreted in both the glucuro- and sulfoconjugated forms while the third 3β-isomer, 19-NEA is sulfoconjugated. Highest levels of these metabolites are found in the first 10 h, 19-NA glucuronide being measured at 240 ng/mL after 2 days and still present after 105 h at 14 ng/mL.

During the 5 days following the administration, the relative amounts of phase I and II metabolites varied constantly, the ratio of 19-NA to 19-NE changing from 2 to around 70. The glucuroconjugated metabolites being excreted more rapidly, 19-NA glucuronide accounted for 95% of the total excreted in the first hours. However, after 6 h the sulfate became predominant (80% of total). As described by other groups [5–7,54], the glucuronide of 19-nortestosterone and of 5β-norandrost-4-en-3,17-dione as well as other metabolites are present but only in the first hours.

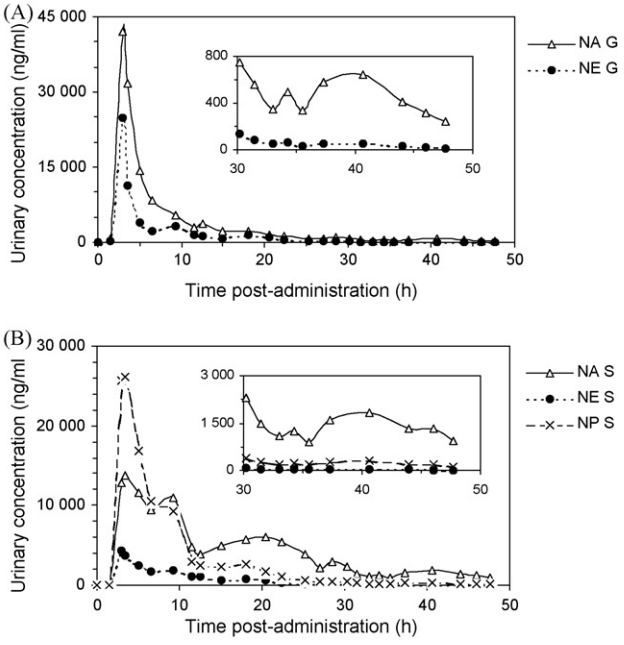

Fig. 1 – 19-norsteroids metabolites excreted in glucuroconjugated (A) and sulfoconjugated (B) forms after oral administration of a single dose of 4-nordione to a volunteer (M26). Concentration in ng/mL corrected for specific gravity of 1.020.

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Table 1 – Relative abundance of NA, NE and NP in positive urine samples collected from athletes.

No sample[a]	Conc. NA G[b] (ng/mL)	% NA G (G/G + S)	% NE G (G/G + S)	% NA NA/Total[c]	% NE NE/Total	% NP NP/Total
M1993A	6.7	90%	100%	79%	10%	12%
M1993B	23.8	77%	89%	71%	21%	8%
M1994	44	79%	98%	75%	19%	7%
M1995	3540	77%	92%	72%	18%	10%
M1997	58	94%	98%	85%	11%	4%
M1998	195	69%	85%	80%	3%	17%
M1999A	5.5	65%	100%	80%	3%	17%
M1999B	36500	73%	89%	60%	24%	15%
M2000A	433	29%	93%	48%	42%	10%
M2000B	3.7	100%	100%	76%	24%	0%

[a] No sample: M = male and No = year of sample collection.
[b] Concentration adjusted to a specific gravity of 1.020.
[c] Total = total amount of glucuro- and sulfoconjugated NA, NE and NP.

3.2. *Athlete's samples containing 19-norandrosterone*

19-NA found in randomly selected positive urine samples was mainly present in the glucuroconjugated form that accounted for 65–93% of the total, the 5β-isomer being also glucuroconjugated to an average of 94%. However, in one specimen collected in 2000, 19-NA sulfate was present in a higher amount than the glucuronide, i.e., 160 and 59 ng/mL respectively. Again, the three isomers were present, 19-NA and 19-NE in both conjugated forms, 19-NEA as the sulfate, in the mean relative proportion of 7:2:1 as summarized in Table 1. A similar ratio of 72:28 was reported previously for the 5α-:5β-isomers [3] although important variations were observed, one specimen containing both metabolites almost equally, and this may reflect the oral ingestion of a 19-norandrostenedione supplement. The sulfates of 19-NE and 19-NEA are not detected when 19-NA is found in an amount lower than 10 and 5 ng/mL.

3.3. *Physiological excretion of norsteroids (pregnancy)*

Fourteen and twenty-six urine samples in the second case, collected regularly from the 10th or 12th week to the 40th week of the pregnancy of 2 volunteers were found to contain norandrosterone in variable amounts reaching a maximum of 15 ng/mL. These results are in agreement with those reported previously [11,15,16]. Noretiocholanolone was only present in low amounts not exceeding 4 ng/mL. Again, norandrosterone was predominantly excreted in its glucuroconjugated form that accounted for 80–94% of the total, the excretion profile in both cases being represented in Fig. 2.

Even though the exact mechanism by which the enzymes of cytochrome P450 convert androgens to estrogens remains to be confirmed, norsteroids were shown to be formed during that process and the placenta is one of the main site of aromatization [19–26,55].

3.4. *Ingestion of edible parts of castrated and non-castrated pig*

No norsteroids were detected in any of the 150 specimens collected from the 19 volunteers, male or female, before the meals or following the ingestion of castrated pig offal. As summarized in Table 2, 19-NA glucuronide was the main norsteroid excreted however in highly variable amounts in the hours following the ingestion of 300 g of mixed non-castrated pig offal and meat. The highest levels of 20–130 ng/mL of 19-NA were reached 5–12 h later and were due to the ingestion of kidney, liver and heart, while only 1 volunteer excreted 19-NA glucuronide in an amount greater than 2 ng/mL after eating 300 g of meat. As expected, the kidneys contain the highest amount of norsteroids. In the specimens collected from the 3 volunteers who ate meals containing kidneys, 19-NA, 19-NE and 19-NEA were measured in both conjugated forms for the formers while the latter was entirely sulfoconjugated. In all the cases, 19-NA was no longer detectable after 20–30 h. Although the levels of excreted 19-norsteroids varied importantly, the

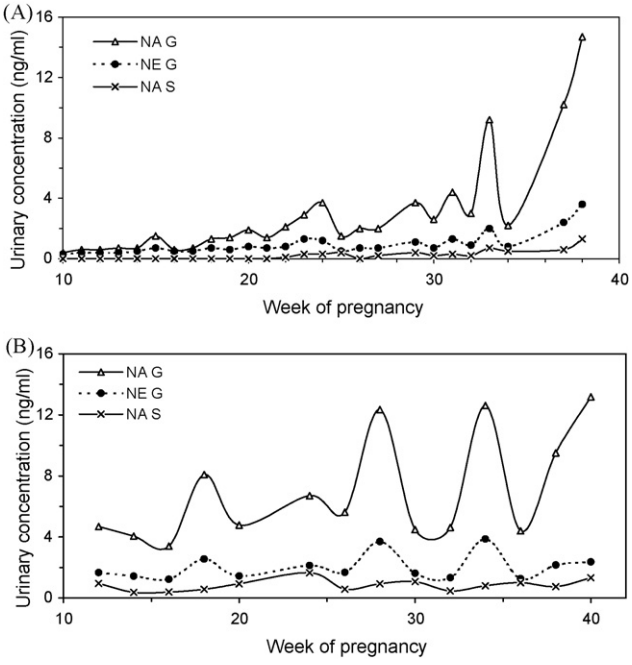

Fig. 2 – Variation of measurable glucuroconjugated and sulfoconjugated 19-norsteroids metabolites excreted during the pregnancy of volunteers W30 (A) and W34 (B). Concentration in ng/mL corrected for specific gravity of 1.020.

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Table 2 – Glucuroconjugated norandrosterone excreted after the intake of non-castrated pig by 12 volunteers.

Volunteer[a]	NA G		Time post-ingestion (h)	Time to return to basal level (h)
	Total excreted (ng/24 h)	Max urinary concentration[b] (ng/mL)		
Kidney, liver, heart mix (300 g)				
M46	31740	62.6	7h00	24h50
M22	11140	21.4	5h30	27h30
W37	19000	130	8h30	30h30
Sarapatel (300 g)				
M23	3500	4.0	5h30	28h00
M25	2040	9.0	4h40	20h15
W36	1330	5.4	2h30	19h45
Meat (300 g)				
M31	880	2.4	3h50	22h10
M37	560	1.7	7h20	23h00
W35	860	0.6	6h10	24h45
Kidney (100 g)				
M24	2860	14.4	5h30	13h16
Liver (100 g)				
M34	530	1.9	5h40	19h30
Meat (100 g)				
M32	193	0.7	3h15	10h45

[a] No sample: M = male, W = woman and No = age of the volunteer.
[b] Urinary concentration adjusted to a specific gravity of 1.020.

excretion profiles were similar. In a typical case presented in Fig. 3, the amount of noretiocholanolone glucuronide was lower in the first hours but became equivalent at the end of the excretion.

When the experiment was repeated with meals containing a normal portion of 100 g, 19-NA glucuronide was excreted in an amount greater than 2 ng/mL only with kidneys (Table 2).

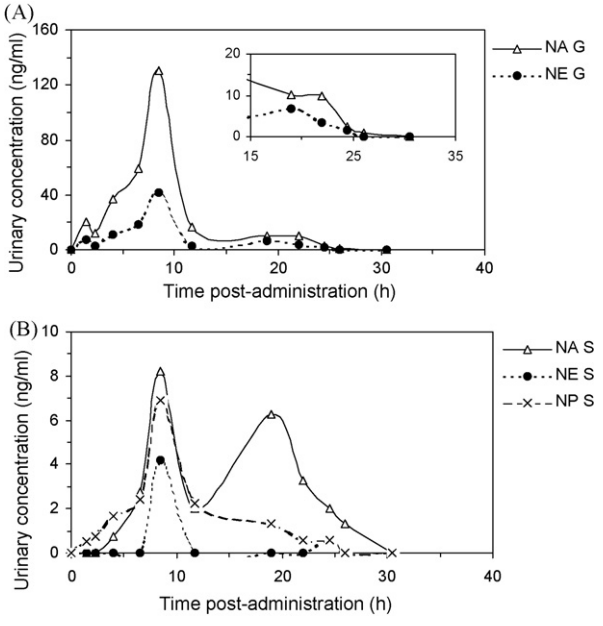

Fig. 3 – 19-norsteroids metabolites excreted after the intake of 300 g of kidney, heart and liver mix from non-castrated pig by a volunteer (W37); glucuronides (A) and sulphates (B). Concentration in ng/mL corrected for specific gravity of 1.020.

The highest levels of 19-nortestosterone and estrogens were measured in the blood of newly born pig during the development of the testis [45]; following castration, these steroids are not detectable anymore in blood and urine specimens [42,56]. While norsteroids are produced naturally by the testis, 19-nortestosterone and 19-norandrostenedione were identified in the μg/kg range in kidneys, liver, heart and muscles [57]. In our experiments, the ingestion of kidneys leads to excreted levels 10–20 times those produced by the consumption of liver and meat respectively. The levels reached were also significantly greater than those reported by another group [49]; this is not unexpected since the levels of norsteroids are known to differ from one animal to another and in function of its age [45,57]. One should however realistically estimate as almost null the risks of ingesting non-castrated pig offal, since only the meat is included in dry meat characteristic of Italian delicatessen while all other products are made from castrated pig.

Finally, the excretion of the norsteroids was not affected by a 1-h work-out period.

3.5. *GC/C/IRMS analysis*

Six specimens from 3 different volunteers having consumed uncastrated pig offal were sent to Cologne for the GC/C/IRMS analysis. The amount of norandrosterone contained in these samples ranged from 20 to 140 ng/mL. The results of the analyses done in duplicate were as follows: the mean ($^{13}C_{VPDB}$ ‰ of 19-NA was measured at −20.93 (−19.77 to −22.43), while the values of references endogenous steroids, androsterone and etiocholanolone were found at −20.58, −20.16 and −20.11

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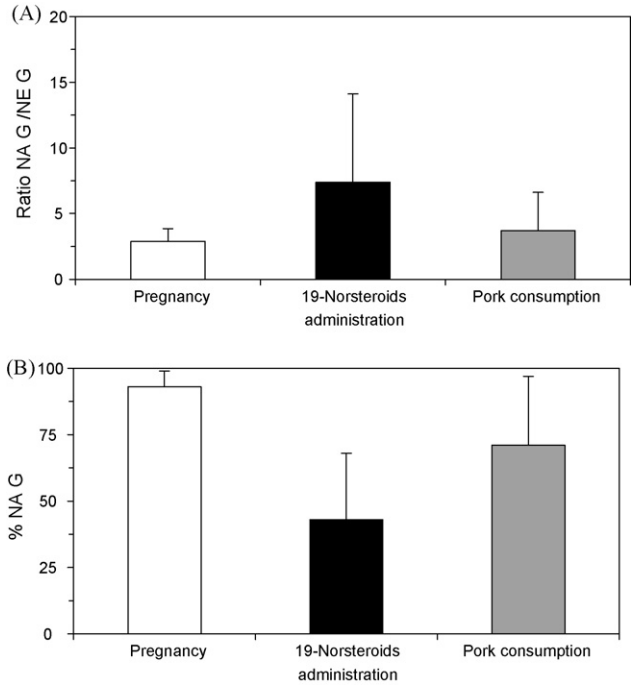

Fig. 4 – Mean values of ratio NA/NE (A) and % NA G (B) for metabolites from endogenous (pregnancy) and exogenous (19-norsteroids administration and consumption of non-castrated pig) origins.

respectively for the 3 volunteers, values that are typical in North Americans.

To the best of our knowledge, this is the first time that the delta ^{13}C values of urinary norsteroids excreted from the ingestion of meat containing 19-norsteroids has been described. These values do not differ from those of several steroids excreted naturally by cows [58–61].

3.6. *Discrimination between endogenous and exogenous origin*

According to our observations, the relative abundance of either the 5α- and 5β-isomers (5α-, 5β-reductases) or of the glucuronides to the sulfates varies widely and cannot serve as basis for establishing their origin in single specific specimen collected at a given moment. The mean value of the ratio 19-NA to 19-NE glucuronides was 3 ± 1 in specimens collected during pregnancy, 7 ± 7 in those collected from athletes or further to the administration of 19-norandrostenedione and 4 ± 3 further to the ingestion of non-castrated pig offal. No more significant were the proportions of the individual conjugates, 19-NA being glucuroconjugated to the mean extent of $93\% \pm 6\%$ (pregnancy), $43\% \pm 25\%$ (administration of norsteroids) and $71\% \pm 26\%$ (non-castrated pig offal) (Fig. 4).

One study reported that 19-NA was excreted solely as the glucuroconjugate following the administration of 19-nortestosterone while up to 30% of 19-NA described as *endogenously* produced, was sulfoconjugated (using *Helix pomatia* mixtures) in the most concentrated of 385 urine samples collected from 40 soccer players [62,63]. These results exemplify the need for an appropriate hydrolysis of conjugated steroids. The arylsulfatases contained in *Helix pomatia* mixtures are known to lack activity for these substrates particularly those conjugated in the 17-O position and of the 5α-H, 3α-ol configuration [64,65]. The chemical solvolysis must be used in place of the enzymatic preparations lacking the activity and specificity needed, and sometimes causing the conversion to other steroids, while in complex matrices, their activity may be inhibited by salts.

4. Conclusion

Our results indicate that when the norsteroids conjugates are properly measured, 19-NA and 19-NE glucuronides and sulfates are present in relative amounts that do not permit a distinction between their synthetic or endogenous origin, which could only be proven by the isotope ratio mass spectrometry [36,37]. With regards to the highly improbable ingestion of non-castrated pig offal outside research context, since norsteroids are normally present in the level of micrograms in the kidneys, liver, heart, not surprisingly, the urine samples collected in the few following hours can contain principally 19-NA glucuronide in an amount that could be in vast excess of the threshold for positivity.

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