RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2001; **15**: 1442–1447

Consequence of boar edible tissue consumption on urinary profiles of nandrolone metabolites. II. Identification and quantification of 19-norsteroids responsible for 19-norandrosterone and 19-noretiocholanolone excretion in human urine

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Received 1 May 2001; Revised 19 June 2001; Accepted 20 June 2001

In previous work (Le Bizec *et al.*, *Rapid Commun. Mass Spectrom.* 2000; 14: 1058), it was demonstrated that a boar meal intake could lead to possible false accusations of abuse of 17β-nortestosterone in antidoping control. The aim of the present study was to identify and quantify endogenous 19-norsteroids in boar edible tissue at concentrations that can alter the steroid urinary profile in humans, and lead to excretion of 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE). The samples were analysed in two laboratories. The methodologies used for extraction and detection (GC/MS(EI) and LC/MS/MS(APCI+)) are compared and discussed. 19-Norandrostenedione (NAED), 17β- and 17α-nortestosterone (bNT, aNT), and 17β- and 17α-testosterone (bT, aT) were quantified. The largest concentrations of NAED and bNT were observed in testicles (83 and 172 μg/kg), liver (17 and 63 μg/kg) and kidney (45 and 38 μg/kg). A correlation between the bNT and NAED content of a typical meal prepared with boar parts and the excreted concentrations of 19-NA and 19-NE in human urine was demonstrated. Copyright © 2001 John Wiley & Sons, Ltd.

17β-Nortestosterone (bNT) is an anabolic androgenic steroid. It is used in human medicine to treat osteoporosis in post-menopausal women and abused in several sports to enhance performance.[1–4] Injectable esters have been used illegally for growth promotion in cattle in the European Union (EU).[5–7] Positive urine samples taken from athletes can be due to unintentional ingestion of anabolic steroids containing meat derived from treated[8,9] or naturally producing animals such as intact boars and stallions.[10] Thus, consumption of boar edible tissue can lead to an altered steroid urinary profile in humans and to false positive results for bNT in antidoping controls.[11] As an extension of our earlier work on bNT, because 19-norsteroid identities and concentrations in boar edible tissues have never been exhaustively considered, the present study focused on structural identity and content of these steroids in various boar matrices.

EXPERIMENTAL

Animals

For this experiment, edible boar tissues were collected. From five intact male boars (2–4 years old, weight 147–186 kg), liver, kidney, muscle tissue, heart and testicles were collected. Portions of liver and kidney that were consumed in the previous experiment[11] were also analysed. The samples were stored frozen (−18 °C) until analysis.

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Reagents and chemicals

The chemicals used for extraction were of analytical grade. The solvents for preparation of the mobile phase were of HPLC-grade. Both were obtained from Merck (Darmstadt, Germany). Most of the steroid standards were obtained from Sigma (St. Louis, MO, USA): 17β-nortestosterone, 19-norandrostenedione, 17β- and 17α-testosterone, 17α-methyltestosterone (external standard), 17β-nortestosterone-d_3 (internal standard). 17α-Nortestosterone was obtained from Steraloids (Newport, Rhode Island, USA). Stock solutions were prepared at 200 ng/μL in ethanol. The working solutions were also prepared in ethanol. Buffer (pH 9) was prepared as a mixture of 2.68 g Na_2HPO_4 and 0.015 g KH_2PO_4 in 500 mL ultrapure water. Subtilisin (0.2 mg; proteinase N from *Bacillus subtilis* 5.6 U/mg, Sigma) was added to 10 mL buffer and prepared as required. The Si-cartridge (500 mg, 10 mL) and NH_2-cartridge (100 mg, 1 mL) were from IST (Hengoed, Mid Glamorgan, UK). *Helix pomatia* enzymatic preparation Biosepra (Villeneuve la Garenne, France) contained both arylsulfatase (20,000 Roy units) and β-glucuronidase (20,000 Fishman units).

DOI:10.1002/rcm.391
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Table 1. Summary of the MS/MS acquisition settings for the LC/MS/MS method

	Analyte	Precursor ion (*m/z*)	Relative collision energy (%)	Product ions (*m/z*)
LC retention time segment 1				
Scan event 1	bNT	275	25	257, 239, 199
Scan event 2	bNT-D_3	278	25	260, 242, 202
Scan event 3	NAED	273	28	197, 237, 255
LC retention time segment 2				
Scan event 1	aNT	275	25	257, 239
Scan event 2	a,bT	289	27	271, 253, 109
Scan event 3	MT	303	27	227, 267, 285
Scan event 4	MT-D3	306	27	230, 270, 288

The derivatisation reagents, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and trimethyliodosilane (TMIS), were purchased from Fluka (Buchs, Switzerland). Dithiothreitol (DTT) was from Aldrich (Milwaukee, WI, USA). The solid-phase extraction (SPE) columns were from SDS (Harbor City, CA, USA) (silica: 1 g, aminopropyl: 0.5 g) and Supelco (St-Quentin-Fallavier, France) (styrene/divinylbenzene copolymer resin, Envi-ChromP: 0.5 g).

Instrumentation

LC/MS/MS

The HPLC apparatus comprised an Agilent 1100 series pump, autosampler and vacuum degasser (Agilent, Palo Alto, CA, USA). Separation was performed on a Symmetry C18 column (5 µm, 150 × 2.1 mm; Waters, Milford, USA). Analysis was carried out using an LCQ^{DECA} ion trap mass analyser (ThermoQuest, San Jose, USA), with an atmospheric pressure chemical ionisation (APCI) interface and XCalibur 1.2. software. The APCI vaporiser temperature was operated at 450 °C, the capillary temperature at 200 °C. The maximum ion time was set to 400 ms, and the discharge current to 10 µA. The analytes were detected in MS/MS full-scan positive ion mode. A summary of the MS/MS acquisition settings is given in Table 1. To separate the different compounds an isocratic mixture of 0.02 M formic acid in methanol and water (45:55, v/v) was used at a flow rate of 0.3 mL min^{-1}.

GC/MS

The quadrupole mass spectrometer used was a model 5973 coupled to a model 6890 gas chromatograph, both from Hewlett Packard (Palo Alto, CA, USA). The split/splitless injector was maintained at 250 °C, the pulse time for the splitless mode was set at 1.5 min, and the injected volume was 3 µL. An OV-1 (Ohio-Valley) capillary column was used for separation (30 m × 0.25 mm i.d., film thickness 0.25 µm). The temperature program used on the OV-1 column was: 120 °C (2 min), 15 °C min^{-1} until 250 °C (0 min), and then 5 °C min^{-1} until 300 °C (8 min). Helium was used as carrier gas at 1 mL min^{-1}. The nominal electron beam energy was set at 70 eV in the electron impact (EI) mode. The mass spectrometric analysis was performed in selected ion monitoring (SIM) mode.

Sample preparation

First approach

Five grams of tissue (liver, kidney, muscle tissue, heart, testicle) were digested with 0.2 mg of subtilisin (2 h, 62 °C, 10 mL buffer pH 9).[13] To precipitate the proteins, 10 mL of methanol were added. The mixture was hand-shaken and vortexed. After centrifugation (20 min, 9000 rpm), the supernatant was transferred through a plug of cotton in a separation funnel. After a wash step with 5 mL of n-hexane the analytes were extracted twice with 20 mL of diethyl ether. The extract was washed with 3 mL ultrapure water and evaporated to dryness using a Rotavap. The residue was resuspended in 500 µL chloroform. The Si-cartridge was conditioned with 5 mL n-hexane and just before application to the cartridge 5 mL n-hexane were added to the sample. After application the sample container was rinsed with 5 mL n-hexane and applied onto the Si-cartridge as well. Another SPE-cartridge (NH_2) was placed underneath the Si-cartridge and both were washed with 5 mL n-hexane. The analytes were eluted three times with 2.5 mL chloroform/acetone (40:10, v/v). The eluant of the first cartridge (Si) was allowed to pass directly onto the other cartridge (NH_2) placed directly beneath the first.[12] The residue was evaporated to dryness using a Speedvac system, and was redissolved in 30 µL methanol and 90 µL 0.02 M formic acid. 60 µL were injected onto the HPLC column.

Second approach[14]

Each tissue sample (15 g) was lyophilised and ground to a powder. Liquid-liquid extraction (LLE), using 12 mL methanol and 15 mL acetate buffer (0.2 M, pH 5.2), resulted in extraction of all forms of the compounds (esters, free and conjugates). After evaporation of the methanol, 80 µL *Helix pomatia* enzymatic preparation were added to the remaining acetate buffer extract, and the mixture was incubated for at least 15 h (52 °C, pH 5.2). After hydrolysis, the extract was transferred onto a copolymeric phase SPE column (ENVI-ChromP), which had previously been conditioned with 6 mL ethyl acetate, 6 mL methanol and 6 mL water. After elution of interfering compounds with 3 mL hexane, the analytes were eluted from the SPE column with 14 mL hexane/diethyl ether (70:30, v/v). After evaporation, ester forms were hydrolysed for 30 min at 50 °C with 500 µL sodium methylate (1% w/v in methanol). Addition of 1 mL sodium hydroxide (1 M) and 8 mL hexane/diethyl ether (70:30, v/v) allowed the separation of phenolic compounds from Δ4-3-one steroids. After elimination of the aqueous phase and evaporation of the organic solvent, the dry residue was resuspended in 500 µL hexane/dichloromethane (60:40, v/v) and applied to a SPE silica column previously conditioned

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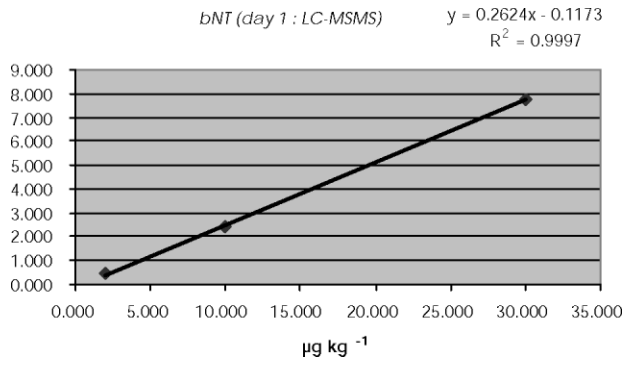

Figure 1. Example of calibration curve for 17β-nortestosterone acquired by LC/MS/MS on day 1.

Table 3. Summary of the recoveries for both extraction procedures

		Recovery (%)	
Day		Subtilisin	*Helix pomatia*
Day 1	Average	44.6	10.7
	Extremes	34–55	9–13
Day 2	Average	42.7	10.5
	Extremes	19–59	7–15
Day 3	Average	46.0	10.7
	Extremes	34–59	9–12
Day 4	Average	57.0	11.6
	Extremes	53–61	7–12

with hexane. The column was washed with 8 mL hexane/ethyl acetate (75:25, v/v). The analytes were eluted with 13 mL hexane/ethyl acetate (60:40, v/v). After evaporation, 15 µL of MSTFA/TMIS/DTT (1000:5:5, v/v/w) were applied to the dry residue, and incubated for 40 min at 60 °C. Two microliters of the derivatized sample were injected.

RESULTS AND DISCUSSION

Intercalibration

17β-Nortestosterone-d_3 was added as an internal standard at the beginning of the extraction procedure, to a concentration of 5 µg/kg. 17α-Methyltestosterone was added as an external standard before the final evaporation step, in each case at the same concentration. Bovine 'blanks' were used from female cows. The calibration curves for liver and kidney were based on spiked 'blank' bovine kidney samples at concentrations of 0, 2, 10 and 30 µg/kg (Fig. 1). The calibration curves for muscle tissue and heart were based on spiked 'blank' bovine muscle tissue samples at concentrations of 0, 2, 4 and 6 µg/kg. Calibration samples were analysed on each day of analysis. Good linearity was obtained for the two techniques used. The coefficients of determination were greater than 0.99 for each analyte. Response factors were calculated for both methods, and are summarized in Table 2. The reproducibility of the response factors is generally acceptable with the exception of results of day 2. Note that the first approach, involving subtilisin and LC/MS/MS, used kidney samples instead of muscle samples. Matrix effects as well as endogenous presence of analytes could be possible explanations for the slightly deviating results. It should be mentioned that for all calculated concentrations the slightly positive responses of the blank value samples were subtracted.

The recoveries for both extraction procedures are summarized in Table 3. Both extraction procedures are repeatable. Recovery yields observed with subtilisin are higher than those obtained with *Helix pomatia*. Nevertheless, we judged the recovery repeatability and the degree of purification to be more critical than the extraction yield itself. Concerning the mass spectrometric analyses, different results were obtained depending on the technique used. In the first approach, using subtilisin, conventional mass spectrometry was not sufficient to filter out non-target compounds, and, in that case, LC/MS/MS was the method of choice (Fig. 2). The signal obtained from the second extraction procedure gave good signal-to noise-ratios at 5 µg kg^{-1} for b-NT and NAED (Fig. 3), and did not show any interference in the blanks (even for liver samples) when conventional single-stage mass spectrometry was used (Fig. 4).

A boar kidney was analysed on three different days using both procedures. The average concentrations and coefficients of variation are summarized in Table 4. The LC/MS/MS procedure (subtilisin extract) showed a slightly better coefficient of variation for 17β-nortestosterone and for 19-norandrostenedione compared to the GC/MS procedure. There is a good similarity between the calculated concentrations of all analytes from the two methods, except for NAED in all matrices and bNT in liver. The alkaline hydrolysis combined with *Helix pomatia*, however, tends to liberate more 19-norandrostenedione than subtilisin. A possible

Table 2. Summary of the response factors for the two techniques

	Technique used				Response factors				
Day	Subtilisin	*Helix pomatia*	LC/MS/MS	GC/MS	NAED	bNT	aNT	bT	aT
Day 1	x		x		0.36	0.24	0.24	0.24	0.24
		x		x	0.16		0.29		
Day 2	x		x		*1.84*	*0.45*	*1.13*	*0.67*	*0.71*
		x		x	*0.11*		0.29		
Day 3	x		x		0.34	0.27	0.36	0.18	0.31
		x		x	0.18		0.43		
Day 4	x		x		0.39	0.26	0.29	0.17	0.29
		x		x	0.17		0.37		

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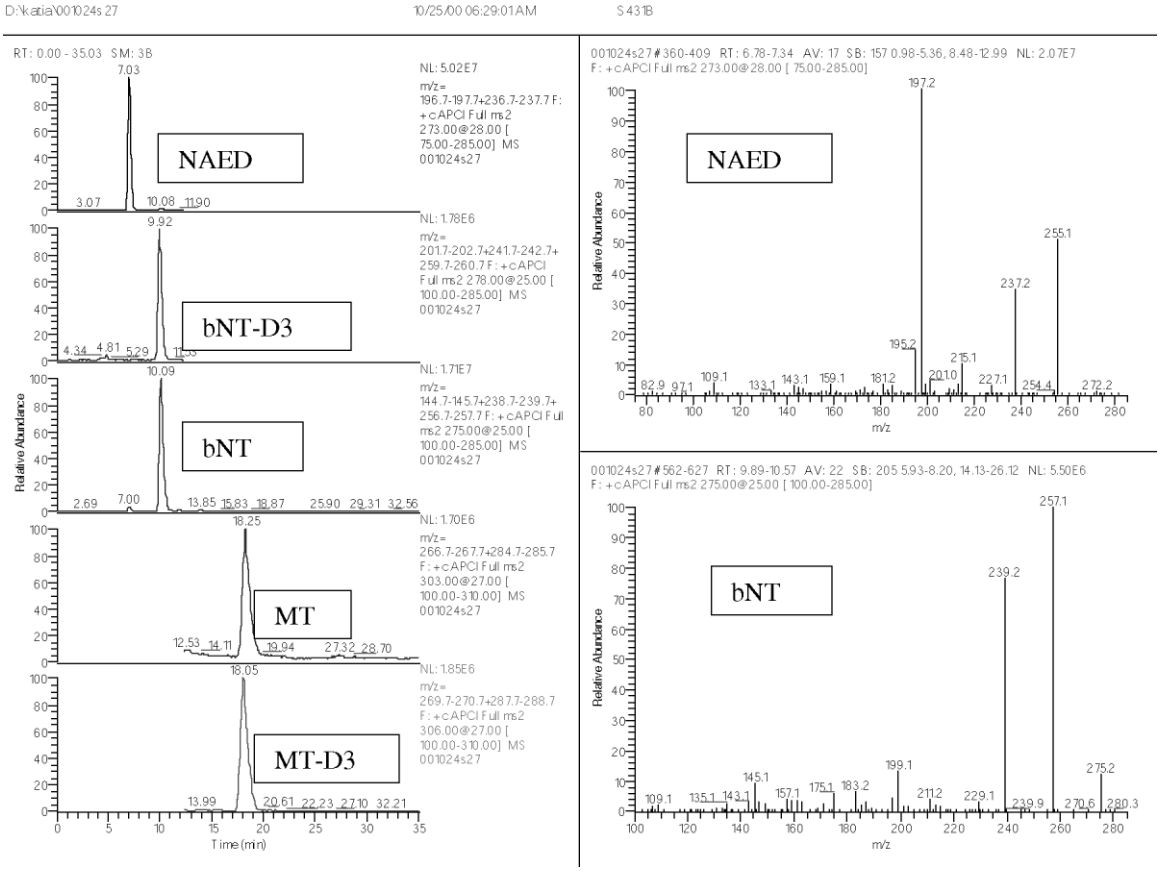

Figure 2. LC/MS/MS ion chromatogram of a positive kidney sample (left) and MS/MS spectrum of 19-norandrostenedione (right, upper) and 17β-nandrolone (right, lower).

explanation is probably linked to the steroid conjugated forms that cannot be hydrolysed by the subtilisin preparation, unlike *Helix pomatia*. Moreover, in the second approach, an alkaline hydrolysis was performed specifically to break all potential ester bonds between steroids and fatty acids. When this step is not applied (*Helix pomatia* alone), the observed NAED concentration is lower than observed using subtilisin. This implies that part of the NAED is covalently attached to another molecule. The increase in the NAED signal observed when subtilisin or alkaline hydrolysis is used means that pH influences the release of NAED conjugates.

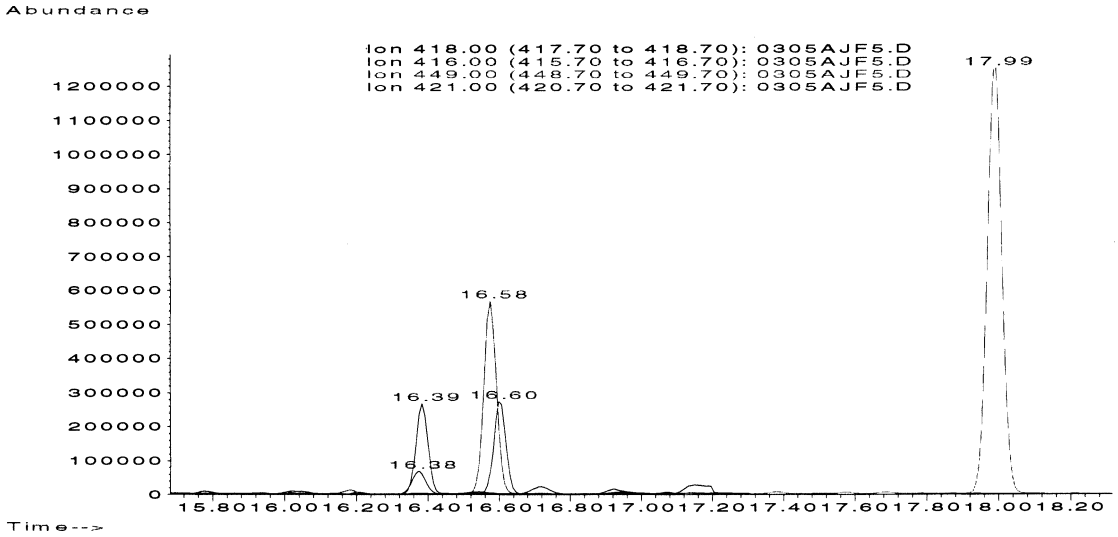

Figure 3. GC/MS ion chromatogram of a 5 ppb spiked liver sample containing 19-norandrostenedione (*m/z* 416, 16.39 min), 19-nortestosterone-d_3 (*m/z* 421, 16.58 min), 19-nortestosterone (*m/z* 418, 16.60 min), 17α-methyltestosterone-d_3 (*m/z* 449, 17.99 min).

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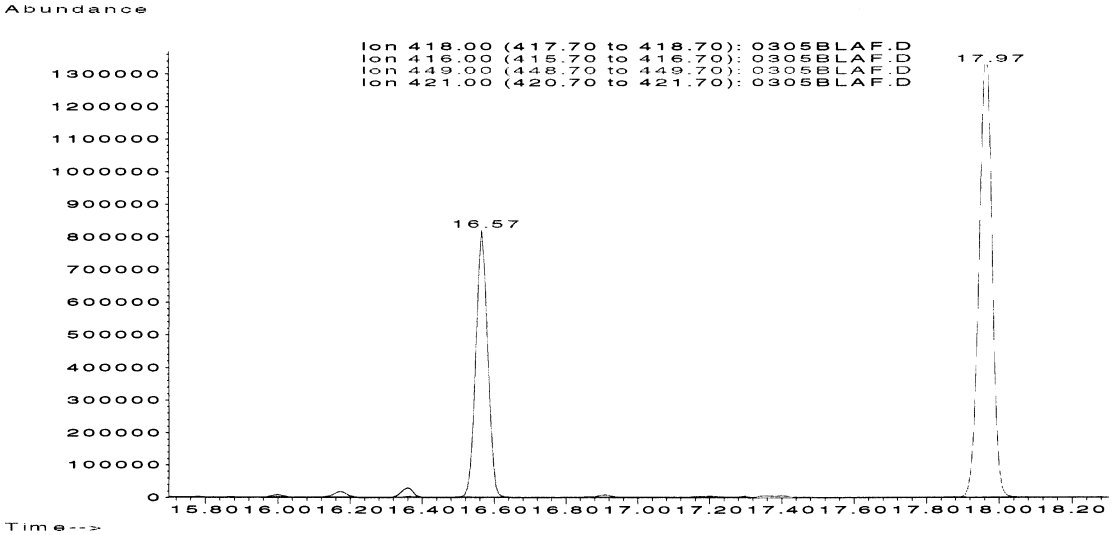

Figure 4. GC/MS ion chromatogram of a blank liver sample spiked with 19-nortestosterone-d_3 (*m/z* 421, 16.57 min) and 17α-methyltestosterone-d_3 (*m/z* 449, 17.97 min).

Table 4. Summary of the average concentrations and coefficients of variation of one kidney analysed over 3 days

	Technique used				Results		
Molecule	Subtilisin	*Helix pomatia*	LC/MS/MS	GC/MS	Average (μg kg^{-1})	Standard deviation (μg kg^{-1})	CV (%)
a-NT	x		x		/	/	/
		x		x	/	/	/
b-NT	x		x		16.1	2.5	18
		x		x	15.8	4.7	30
NAED	x		x		18.5	2.0	11
		x		x	36.9	4.8	13
a-T	x		x		/	/	/
		x		x	/	/	/
b-T	x		x		8.6	2.4	28
		x		x	9.7	2.5	27

Table 5. Summary of the concentration ranges in the different matrices

	Technique used				Molecules	
Matrix	Subtilisin	*Helix pomatia*	LC/MS/MS	GC/MS	NAED (μg kg^{-1})	*β*-NT (μg kg^{-1})
Liver	x		x		1.0 → 5.0	0.4 → 35.0
		x		x	1.3 → 17.4	0.9 → 62.8
Kidney	x		x		0.9 → 20.0	1.1 → 19.0
		x		x	0.7 → 45.5	0.5 → 19.5
Heart	x		x		0.6	0.8 → 2.0
		x		x	/	0.3 → 8.5
Testicle	x		x		1.5 → 48.0	7 → 131.0
		x		x	3.6 → 83.5	12.9 → 172.1
Meat	x		x		0.6 → 0.9	1 → 2.5
		x		x	0.5	3.6
Kidney*	x		x		81.0	38.0
		x		x	86.6	34.3
Liver*	x		x		2.1	9.8
		x		x	3.1	17.6

* Edible tissues from our previous experiment.[11]

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Table 6. Calculation of excreted 19-NA and 19-NE in the three individuals (Dirk, Kurt and Luc) of our previous experiment.[11] For each volunteer, the average 19-NA and 19-NE concentrations are given, the average excreted quantity in μg for each metabolite and the total 19-NA equivalent quantity in μg based on a 1.5 L urinary excretion during one day

Time	Dirk		Kurt		Luc	
Sampling time	[NA] ng/mL	[NE] ng/mL	[NA] ng/mL	[NE] ng/mL	[NA] ng/mL	[NE] ng/mL
Average excreted metabolite (ng/mL)	2.79	0.53	1.56	0.21	1.36	0.54
Excreted metabolite (μg) for an average of 1.5 L urine/day	4.18	0.79	2.35	0.31	2.03	0.81
Total 19-NA equivalent (μg) for an average of 1.5 L urine/day	4.98		2.66		2.84	

17β-Nortestosterone and 19-norandrostenedione concentrations in different matrices

Table 5 summarizes the concentrations in the different matrices. The highest concentrations of 17β-nortestosterone and 19-norandrostenedione were found in testicles, liver and kidney (Fig. 2). Meat and heart contained the lowest amounts of both compounds. Referring to our previous work on the influence of boar tissue consumption on 19-NA and 19-NE urinary excretion in humans, a good correlation was observed between NAED and bNT intake from a boar meal and 19-NA and 19-NE released in human urine. On the one hand, a simple calculation of the total intake of NAED and bNT from the boar meal consumed by the three volunteers,[11] i.e. 90 g kidney (81 μg/kg NAED and 38 μg/kg bNT) and 90 g liver (2 μg/kg NAED and 10 μg/kg bNT)), leads to 11.9 μg theoretical 'nandrolone equivalent'. In this calculation the contribution of meat and heart was disregarded because of their negligible NAED and bNT content. As 100% of the ingested 19-norsteroids was eliminated within 24 h in urine,[11] the total urinary quantity of combined metabolites should be 11.9 μg as well. On the other hand, the observed average excreted quantity in urine was calculated taking into account the measured 19-NA and 19-NE concentrations multiplied by an average excreted urine volume of 1.5 L per day. For volunteer Dirk, 5 μg of total 19-NA equivalents were measured, and for Kurt 2.7 μg and for Luc 2.8 μg (Table 6). The absolute amounts measured were based on an average excretion of metabolites. However the absolute amount per excretion can vary depending on the volume excreted. Part of the difference between the theoretical 11.9 μg and the measured amounts is due to that fact. However, it is also important to mention that losses can occur linked to faecal elimination of 19-NA and 19-NE, to possible storage in fat tissues, and to all the mathematical approximations used in this estimation. Nevertheless, the 19-NA and 19-NE quantities excreted in urine are clearly of the order of the estimated absorbed amounts of b-NT and NAED.

CONCLUSIONS

Although the recoveries of the two extraction methods differ, the quantitative results for the compounds of interest are similar in both laboratories using the two different approaches. Using digestion with subtilisin, the amount of 17β-nortestosterone was found to almost equal the amount of 19-norandrostenedione present in the different matrices. Alkaline hydrolysis combined with *Helix pomatia* allows the detection of a larger amount of 19-norandrostenedione whatever the matrix, and of 17β-nandrolone especially in liver. The greatest concentrations of 19-norandrostenedione and 17β-nortestostosterone were observed in testicles (83 and 172 μg/kg), liver (17 and 63 μg/kg) and kidney (45 and 38 μg/kg). Referring to the previous experiment in which a meal consisting of kidney, liver, heart and meat was consumed by three volunteers, differences based on the averaging of data and some necessary assumptions were observed between the 19-norsteroid intake and the 19-NA and 19-NE excreted in human urine.

Acknowledgements

The authors wish to thank Wendy Derycke and Mieke Naessens for their skilful operation of the LC/MS/MS apparatus and sample preparation. We are grateful to Isabelle Gaudin and Christel Gade for their help in steroid preparation and GC/MS analysis, and Frédéric Poulain for his expertise and advices concerning the quality management of this project.

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