Urinary metabolic profile of 19-norsteroids in humans: glucuronide and sulphate conjugates after oral administration of 19-nor-4-androstenediol

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Received 25 March 2008; Revised 17 July 2008; Accepted 19 July 2008

19-Nor-4-androstenediol (NOL) is a prohormone of nandrolone (ND). Both substances are included in the WADA List of Prohibited Classes of Substances and their administration is determined by the presence of 19-norandrosterone (NA) with the urinary threshold concentration of 2 ng mL−1. Routine analytical procedures allow the determination of NA excreted free and conjugated with glucuronic acid, but amounts of ND and NOL metabolites are also excreted in the sulphate fraction. The aim of this study is to determine the urinary metabolic profile after oral administration of a nutritional supplement containing NOL. Urine samples were collected up to 96 h following supplement administration and were extracted to obtain separately three metabolic fractions: free, glucuronide and sulphate. Extraction with tert-butyl methyl ether was performed after the hydrolysis steps and trimethylsilyl derivatives were analyzed by gas chromatography/mass spectrometry (GC/MS). After oral administration of NOL, the main metabolites detected were NA and noretiocholanolone (NE) in the glucuronide and sulphate fractions. The relative abundances of each metabolite in each fraction fluctuate with time; a few hours after administration the main metabolite was NA glucuronide whereas in the last sample (4 days after administration) the main metabolite was the NA sulphate and the second was the NE glucuronide. During the studied period almost half of the dose was excreted and the main metabolites were still found in urine after 96 h. Norepiandrosterone and norepietiocholanolone were also detected only in the sulphate fraction. Our results suggest that sulphate metabolites should be taken into consideration in order to increase the retrospectivity in the detection of 19-norsteroids after oral administration. Copyright © 2008 John Wiley & Sons, Ltd.

Nandrolone (ND) is an anabolic-androgenic steroid, similar to the endogenous hormone testosterone, that promotes muscle growth. For this reason, it is considered as a performance-enhancing drug and is included by the World Anti-Doping Agency (WADA) in the list of banned substances in sport.1 ND is one of the most detected drugs in doping control in sports.2 Orally administered ND and other 19-norsteroids are metabolized to 19-norandrosterone (NA), 19-noretiocholanolone (NE) and 19-norepiandrosterone (ENA) (Fig. 1). The first two are predominantly excreted in urine as glucuronide and sulphate derivatives, while the latter was reported as being conjugated with sulphate.3 Illegal use of ND is detected by the presence of NA with the urinary threshold concentration of 2 ng·mL−1, normally measured in the free plus glucuronide fractions.4 The limit was set because NA can be present at low concentrations in the urine of males and females as an endogenous substance.5–11 In addition, formation of NA from endogenous androsterone during sample storage has recently been demonstrated.12 The consumption of boar meat has also been reported as an unintentional source of NA in urine,13,14 and the consumption of nutritional supplements contaminated with prohormones can result in positive doping cases.15–25 Prohormones of ND, such as 19-norandrostenedione (NONE), 19-nor-5-androstenediol and 19-nor-4-androstenediol (NOL), have been synthesized as precursors of ND and can be applied via oral, transdermal or sublingual administration. They were considered nutritional supplements and were sold as over-the-counter products in the USA as a result of the ‘Dietary Supplement and Health Education Act of 1994’.26 However, they were included in the list of anabolic steroids forbidden in sports in 1999 by the International Olympic Committee (IOC), and they remain on the current WADA list. Their illegal use is demonstrated by the detection of NA in urine. Legislative classification has changed in the USA and currently they are controlled substances (Anabolic Steroid Control Act of 2004).27

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Contract/grant sponsor: Ministerio de Educación y Ciencia; Consejo Superior de Deportes (Spain); Departament d’Universitats, Recerca i Societat de l’informació; and Consell Català de l’Esport (Catalan).
Routine analytical procedures in doping control laboratories allow the determination of NA excreted free and conjugated with glucuronic acid in urine, but amounts of ND and NOL metabolites are also excreted in the sulphate fraction. Among previous papers dealing with the metabolism of norsteroids in human urine, some have discussed the sulphate fraction. Le Bizec and co-workers suggested that urinary NA formed after ND administration is exclusively conjugated to glucuronic acid whereas a proportion (\( \approx 30\% \)) of that produced from an endogenous source is sulpho-conjugated. Another recent study investigated the urinary conjugates of the major ND metabolites (NA, NE and ENA) after oral administration of 19-norsteroids. The study confirmed that NA and NE were present as both the glucuronide and the sulphate conjugates, and ENA was excreted exclusively as a sulphate fraction in the urine of all samples tested. The sulphate fraction is also important in the norsteroid metabolism of other species, such as boar and horse.

The aim of the present work was to study the metabolic profile of NOL in urine after oral administration of the doses specified by the manufacturer. The concentrations of NOL and its major and minor metabolites have been quantified with a method that allows us to discriminate between the free, glucuronide and sulphate fractions of metabolites.

**EXPERIMENTAL**

**Chemical and reagents**

19-Norandrosterone (3a-hydroxy-5a-estran-17-one, NA), 19-noretiocholanolone (3a-hydroxy-5β-estran-17-one, NE), methyltestosterone (17β-methyl-4-androsten-17β-ol-3-one), NA glucuronide, NE glucuronide, nandrolone glucuronide and 19-norandrosterone-\( d_4 \) ([2,2,4,4-\( ^2 \)H\(_4\)]-3α-hydroxy-5α-estran-17-one) were supplied by NMI (Pymble, Australia); 17β-nortestosterone (4-estren-17β-ol-3-one, ND) was supplied by Sigma (St. Louis, MO, USA); 19-nor-4-androstenediol (4-estren-3β,17β-diol, NOL), 19-norepiandrosterone (3β-hydroxy-5α-estran-17-one, ENA) and 19-norepietiocholanolone (3β-hydroxy-5α-estran-17-one, ENE) were purchased from Steraloids Inc. (Newport RI, USA); and 19-norandrostenedione (4-estren-3,17-one, NONE) was supplied by Research Plus Inc. (Manasquan, NJ, USA).

Methanol, ethyl acetate (HPLC grade), disodium hydrogen phosphate, sodium hydrogen phosphate, tert-butylmethyl ether (TBME), sodium hydroxide pellets, ammonium iodide, 2-mercaptopethanol, potassium carbonate (all analytical grade) and sulphuric acid (extra pure grade) were purchased from Merck (Darmstadt, Germany). β-Glucuronidase from *Escherichia coli* K12 was provided by Roche Diagnostics GmbH (Mannheim, Germany). N-Methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Düren, Germany). Ultra-pure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Detectabuse™ solid-phase extraction (SPE) columns (XAD-2) were purchased from Biochemical Diagnostics Inc. (New York, NY, USA) and Sep Pak C18 Vac RC cartridges (500 mg) were supplied by Waters (Milford, MA, USA). Bulk human blank urine was supplied by BioRad (Hercules, CA, USA).

**Standard solutions**

Separate stock standard solutions (1 mg mL\(^{-1}\), in free base form) of each analyte were prepared using methanol as solvent. Successive 1:10 dilutions with methanol of the 1 mg mL\(^{-1}\) stock solutions were made to prepare working solutions of 100, 10, 1 and 0.1 μg mL\(^{-1}\). All solutions were stored at \(-20^\circ\) C.

**Sample preparation**

**Free fraction extraction**

To 5 mL urine samples, internal standard (ISTD) solutions (20 μL of the 4 μg mL\(^{-1}\) 19-norandrosterone-\( d_4 \) and methyltestosterone solutions) were added. Then, the samples were adjusted to pH 7 with 1.5 mL of sodium phosphate buffer (0.2 M, pH 7). The mixture was extracted with 5 mL of TBME by rocking at 40 movements/min for 20 min. After centrifugation (2000 g, 5 min), the organic layers were separated and evaporated to dryness under a stream of nitrogen in a water
bath at 40°C. The extracts were kept in desiccators containing P₂O₅ and maintained under vacuum for at least 30 min before derivatization.

**Glucuronide fraction extraction**

The free fraction was extracted from the samples (5 mL) as described above. The small volume of organic solvent still present on top of the aqueous phase was evaporated under a stream of nitrogen. To the aqueous phase, ISTD solutions (like in the free fraction) were added. The samples were then applied to Sep Pak C18 cartridges previously conditioned with methanol (2 mL) and water (2 mL); the cartridges were washed with water (2 mL) and analytes were eluted with methanol (2 mL). The organic extracts were evaporated to dryness under a stream of nitrogen in a bath at 40°C and reconstituted with 1 mL of sodium phosphate buffer (0.2 M, pH 7). Enzymatic hydrolysis was performed by adding 30 μL of β-glucuronidase from *E. coli* and incubating the mixture at 30°C for 30 min before derivatization. Minimum cleavage of the derivatized extracts were transferred to injection vials and analyzed by GC/MS.

**Sulphate fraction extraction**

The samples were treated as described for the glucuronide fraction up to the SPE step. The extracts obtained after Sep Pak C18 extraction were reconstituted with 4 mL of ethyl acetate/methanol/sulphuric acid (80:20:0.06, v/v/v) and incubated at 55°C for 2 h. The samples were then neutralized with 60 μL of 1 M NaOH and evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 1 mL of sodium phosphate buffer (0.2 M, pH 7) and 250 μL of 5% K₂CO₃ solution were then added. The mixture was extracted with 5 mL of TBME by rocking at 40 movements/min for 20 min. After centrifugation (2000 g, 5 min), the organic layers were separated and evaporated to dryness under a stream of nitrogen in a water bath at 40°C. The extracts were kept in desiccators until derivatization using the same conditions as for the free fraction.

**Derivatization**

The dry extracts were derivatized by adding 50 μL of a mixture of MSTFA/NH₄OH/2-mercaptoethanol (1000:2:6, v/v/w) and incubating at 60°C for 20 min. After incubation, the derivatized extracts were transferred to injection vials and analyzed by GC/MS.

**Chromatographic conditions**

An Agilent (Palo Alto, CA, USA) 6890N GC system equipped with an autosampler (Agilent 7683 series injector) and connected to a quadrupole mass spectrometer (Agilent 5973 mass-selective detector) was used. Separation was performed using a 100% methylsilicone fused-silica capillary column (HP Ultra-1; 17 m × 0.2 mm i.d.; film thickness, 0.11 μm).

Helium was used as the carrier gas at a flow rate of 0.8 mL min⁻¹ (measured at 180°C). The oven was set at an initial temperature of 180°C, and then the following rates were programmed: at 3°C min⁻¹ from 180°C to 230°C, then at 40°C min⁻¹ to 310°C where it was held for 3 min. The injection (2 μL) was performed in split mode (10:1). The injector and the detector were set at 280°C. The mass spectrometer was operated in electron ionization (EI) mode (70 eV) and in selected ion monitoring acquisition mode. Three diagnostic ions were monitored for each compound of interest (Table 1).

**Calibration and quality control samples**

For each batch of samples and for each fraction, calibration curves with five concentration levels (Table 2) in duplicate and quality control (QC) samples containing two different concentrations of each analyte in triplicate were prepared. Calibration samples were prepared daily by adding appropriate volumes of working solutions to blank urine. QC samples were prepared in batches by adding appropriate volumes of working solutions to blank urine; each batch was distributed into aliquots of 5 mL and they were kept at –20°C until testing. Peak area ratios between the selected ion of the analyte and the selected ion of the ISTD 19-norandrosterone-d₄ (after area correction due to the contribution of isotopic ion of NA) were used for calculations. Further dilutions of the urine samples were made when necessary to fit on the calibration curve.

**Method validation**

The following parameters were evaluated during the validation of the analytical method for the free, glucuronide

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**Table 1. Diagnostic ions used for identification and quantification, and absolute and relative retention times (RT and RRT) for the bis-O-TMS derivatives of the analytes under study.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Diagnostic ions (m/z)</th>
<th>RT (min)</th>
<th>RRT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M⁺ M-15 M-90 M-105</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norandrosterone-d₄</td>
<td>424 409 319</td>
<td>8.9</td>
<td>0.59</td>
</tr>
<tr>
<td>Norandrosterone (NA)</td>
<td>420 405 315</td>
<td>8.9</td>
<td>0.59</td>
</tr>
<tr>
<td>Norepiandrosterone</td>
<td>420 405 315</td>
<td>9.7</td>
<td>0.65</td>
</tr>
<tr>
<td>Norepiandrosterone</td>
<td>420 405 315</td>
<td>9.9</td>
<td>0.66</td>
</tr>
<tr>
<td>Norepiandrosterone</td>
<td>420 405 315</td>
<td>10.2</td>
<td>0.69</td>
</tr>
<tr>
<td>Norandrostenediol</td>
<td>420 405 330</td>
<td>11.1</td>
<td>0.74</td>
</tr>
<tr>
<td>Norandrostenediol</td>
<td>416 401 311</td>
<td>11.9</td>
<td>0.79</td>
</tr>
<tr>
<td>Nandrolone (ND)</td>
<td>418 403 194</td>
<td>12.3</td>
<td>0.82</td>
</tr>
<tr>
<td>Methyltestosterone</td>
<td>446 301 14.9</td>
<td>14.9</td>
<td>1.00</td>
</tr>
</tbody>
</table>
and sulphate fractions: linearity, limits of detection (LOD) and quantification (LOQ), extraction recovery, intra- and inter-assay precision, as well as accuracy. Contribution of glucuronides in the sulphate fraction was experimentally evaluated.

The LOD and LOQ were calculated to be 3.3 and 10 times the noise level, respectively. The noise level was set to be the equivalent of the standard deviation (SD) calculated for the lowest calibration level.

Extraction recoveries of each analyte were calculated by comparison of peak areas of the compounds obtained after the analysis of samples spiked with 20 ng mL\(^{-1}\) of each compound (n = 4) with the mean values of those obtained when the standards were added to extracted blank human urine and subjected to the derivatization process (representing 100% of extraction recovery).

Precision and accuracy were determined by comparing the results of QC samples analyzed the same day and on three different days. Precision was expressed as the relative standard deviation (RSD) of the control samples concentration calculated using the calibration curve and accuracy was expressed as the relative error (%) of the estimated concentration.

Potential cleavage of glucuronides in the sulphate fraction was measured by comparison of peak areas of NA, NE and ND glucuronides in the sulphate fraction after sulphate fraction extraction of samples spiked with 100 ng mL\(^{-1}\) of NA glucuronide, NE glucuronide, ND glucuronide (n = 4) with the mean values of those obtained when the equivalent concentration of the free standards was extracted (representing 100% of hydrolysis).

**Clinical protocol and sample collection**

Administration studies of NOL to six healthy male volunteers were performed. The mean age of the participants was 27.5 years (range, 21–37 years), the mean weight was 74.3 kg (range, 62.3–81.8 kg), and the mean height was 177.9 cm (range, 160–187 cm). All participants gave written informed consent before inclusion in the study. The study was conducted in accordance with the Declaration of Helsinki, and following a protocol approved by the local Ethical Committee (CEIC-IMAS 2002/1519), and authorized by the Agencia Española del Medicamento y Productos Sanitarios (reference AEMPS 04-0012) of the Spanish Ministry of Health.

Two capsules of Norandrodiol Select 300 were orally administered. The content of NOL in the nutritional supplement Norandrodiol Select 300 (Ergopharm, Cham-paign, IL, USA) was estimated in house\(^3^8\) as 90.7 ± 11.2 mg per capsule, representing 60.5% of the theoretical content indicated in the label.

Urine samples were collected before administration and at 0–2, 2–4, 4–8, 8–16, 16–24, 24–48, 48–72 and 72–96 h intervals. Sample pH and specific gravity were measured and the samples were stored at −20°C until analysis.

**RESULTS**

**Validation of the analytical method**

The analytical method was optimized for the quantification of NOL and its metabolites in human urine in three metabolic fractions, free, glucuronide and sulphate.

The ions monitored for each compound are listed in Table 1. Depending on the steroid, the ions monitored were the EI molecular ion, the ion resulting from the loss of a methyl group (−15 Da), the ion resulting from the loss of a HOTMS group (−90 Da), the ion resulting from losses of a methyl and a HOTMS group (−105 Da), or other specific fragment ions of the steroids (e.g., m/z 194).

The method was validated to determine linearity, extraction recovery, limit of detection and quantification, intra and inter-day precisions and accuracies. The heteroscedasticity of the procedure was detected by Levene’s test, so a proportional weighted (1/concentration) least-squares regression analysis was selected as the calibration model. Determination coefficients (r\(^2\)) were better than 0.99 in all calibrations. The F test for comparison of variances was not significant, indicating adequate adjustment of the data to the proposed linear model over the calibration range.

Limits of quantification and extraction recoveries obtained in the different metabolic fractions are listed in Table 2. Results obtained for intra- and inter-assay precision and accuracy are presented in Table 3. As can be observed, the values did not exceed 20% in most cases and they were always lower than 28% for all compounds at the studied concentrations.

The stability of glucuronides during sulphate fraction extraction was studied. The percentages of hydrolysis of NA, NE and ND glucuronides in the sulphate fraction were 3.22% (SD: 0.35%), 5.12% (SD: 0.47%) and 2.63% (SD: 0.26%), respectively (n = 4). Metabolite concentrations in the sulphate fraction were corrected according to these results.

**Metabolic profile**

Mean, standard deviation, minimum and maximum concentrations of NOL and its metabolites in the free,
The main metabolites detected in the glucuronide fraction were NA and NE. Both metabolites were still present 96 h after administration. Peak concentrations for NA were found before 4 h post-administration and ranged from 9711.6 to 60936 ng mL$^{-1}$. The time–concentration profiles of NE peaked between 2 and 24 h post-administration and ranged from 6623.8 to 36632 ng mL$^{-1}$. ND glucuronide was detected in four volunteers during the whole collection period, and in the other two volunteers it was found up to 8 h and 24 h. Low concentrations of NOL could be detected in the glucuronide fraction of all volunteers, except in the last two urines of one volunteer.

The main metabolite detected in the sulphate fraction was NA. Important concentrations of NE and ENA were found and the presence of ENE was also detected. The time–concentration profiles of NA, NE and ENA peaked between 2 and 24 h post-administration and the maximum concentrations ranged from 10562 to 50794, 1070 to 17017 and 3887 to 40229 ng mL$^{-1}$, respectively. A peculiar profile was observed for ENE. It was not detected in the first samples after administration, and appeared in urine between 4 and 8 h post-administration for one volunteer, between 8 and 16 h for two other volunteers and after 16 h it was detected in the urines of all volunteers, with maximum concentrations between 4 and 24 h in the range between 56 and 598 ng mL$^{-1}$.

In the free fraction, ND, NA, NE, NOL and NONE were detected at low concentrations in all the urines collected, except for NA that was not detected in the last urine of one volunteer and NOL that was not detected in the last two urines. The time–concentration profiles of NA and NE peaked between 2 and 48 h post-administration, while the maximum concentration of ND, NOL and NONE was before 4 h post-administration.

Figure 2 shows the profile of the mean concentrations of the main metabolites. A few hours after administration the main metabolite is NA glucuronide and in the last sample (4 days after administration) the main metabolite is the NA sulphate, followed by the NE glucuronide.

The profiles of the cumulative amounts of the metabolites excreted in each fraction are shown in Fig. 3. Just after administration, all the metabolites are excreted quickly, except for ENE. The excretion rate stabilizes after 24 h for all the metabolites except for NE in the three fractions and NA in the sulphate fraction, where an increase until 48 h is observed.

The averaged total percentage of the dose administered excreted in urine was calculated taking into account the total cumulative amount of each metabolite in each fraction.
The results showed that a mean of 45.8% of the dose was excreted after 96 h. The percentage of the dose excreted of each metabolite in the different metabolic fractions over all studied time period is also indicated in Table 5.

**DISCUSSION**

The analytical method used in this study allows the quantification of NOL and its metabolites in human urine in three metabolic fractions: free, glucuronide and sulphate. The method has been demonstrated to comply with the criteria for the validation of quantitative methods established according to the requirements of different international organizations and regulatory authorities.39–41 Additional

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**Table 4.** Concentrations detected for each metabolite in each fraction (n a: number of volunteers in which the metabolite was detected)

<table>
<thead>
<tr>
<th>Hours</th>
<th>NA (ng mL⁻¹)</th>
<th>NE (ng mL⁻¹)</th>
<th>ND (ng mL⁻¹)</th>
<th>NOL (ng mL⁻¹)</th>
<th>NONE (ng mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
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</tr>
<tr>
<td>2</td>
<td>6 3.4</td>
<td>0.6–8.5</td>
<td>6 14.5</td>
<td>1.7–44.1</td>
<td>6 1.8</td>
</tr>
<tr>
<td>4</td>
<td>6 9.8</td>
<td>2.1–32.1</td>
<td>6 28.2</td>
<td>4.0–97.2</td>
<td>6 2.6</td>
</tr>
<tr>
<td>8</td>
<td>6 3.8</td>
<td>1.4–9.8</td>
<td>6 6.6</td>
<td>3.0–12.8</td>
<td>6 1.0</td>
</tr>
<tr>
<td>16</td>
<td>6 3.7</td>
<td>0.9–6.7</td>
<td>6 14.4</td>
<td>2.8–46.9</td>
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<td>24</td>
<td>6 4.1</td>
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<td>6 31.8</td>
<td>2.6–69.8</td>
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<tr>
<td>48</td>
<td>6 1.1</td>
<td>0.3–2.1</td>
<td>6 13.2</td>
<td>3.6–29.4</td>
<td>6 0.4</td>
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<tr>
<td>72</td>
<td>6 0.4</td>
<td>0.2–1.1</td>
<td>6 3.8</td>
<td>0.5–15.1</td>
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<tr>
<td>96</td>
<td>5 0.4</td>
<td>0.2–1.7</td>
<td>6 2.5</td>
<td>0.2–8.3</td>
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**Glucuronide fraction**

<table>
<thead>
<tr>
<th>Hours</th>
<th>NA (ng mL⁻¹)</th>
<th>NE (ng mL⁻¹)</th>
<th>ND (ng mL⁻¹)</th>
<th>NOL (ng mL⁻¹)</th>
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<tr>
<td>2</td>
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<td>2242.2–28562.2</td>
<td>6 6898.8</td>
<td>1633.7–18124.5</td>
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<tr>
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<td>6 25671.2</td>
<td>971.6–60936.3</td>
<td>6 8677.2</td>
<td>2934.6–25128.2</td>
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<td>5817.5–17814.3</td>
<td>6 4031.6</td>
<td>1051.7–9673.2</td>
<td>6 173.3</td>
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<td>6 10707.6</td>
<td>2347.6–18551.2</td>
<td>6 10479.8</td>
<td>3786.8–36329.2</td>
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<tr>
<td>24</td>
<td>6 5664.4</td>
<td>997.1–11706.5</td>
<td>6 11536.0</td>
<td>494.8–29977.9</td>
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<td>6 797.6</td>
<td>55.8–2666.3</td>
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<td>5 146.7</td>
<td>6.0–437.9</td>
<td>6 412.8</td>
<td>4.3–1545.3</td>
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**Sulphate fraction**

<table>
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<tr>
<th>Hours</th>
<th>NA (ng mL⁻¹)</th>
<th>NE (ng mL⁻¹)</th>
<th>ENA (ng mL⁻¹)</th>
<th>ENE (ng mL⁻¹)</th>
<th>NOL (ng mL⁻¹)</th>
<th>NONE (ng mL⁻¹)</th>
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<td>6 6499.8</td>
<td>275.5–12821.8</td>
<td>6 2596.8</td>
<td>247.3–5440.2</td>
<td>6 7189.9</td>
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</tr>
<tr>
<td>4</td>
<td>6 22888.7</td>
<td>4168.2–50793.8</td>
<td>6 5690.9</td>
<td>495.1–16509.9</td>
<td>6 17394.0</td>
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</tr>
<tr>
<td>8</td>
<td>6 14905.0</td>
<td>5276.3–25880.7</td>
<td>6 2225.7</td>
<td>569.2–4399.2</td>
<td>6 6976.9</td>
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<tr>
<td>16</td>
<td>6 16589.0</td>
<td>9624.7–26966.3</td>
<td>6 4084.6</td>
<td>415.5–12308.9</td>
<td>6 4418.8</td>
<td></td>
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<tr>
<td>24</td>
<td>6 12450.2</td>
<td>1965.4–32320.2</td>
<td>6 4861.4</td>
<td>551.1–14251.0</td>
<td>6 2506.3</td>
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<tr>
<td>48</td>
<td>6 3197.3</td>
<td>1785.2–67180</td>
<td>6 1967.3</td>
<td>133.9–4448.6</td>
<td>6 412.3</td>
<td></td>
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<tr>
<td>72</td>
<td>6 695.7</td>
<td>271.5–1520.5</td>
<td>6 345.9</td>
<td>25.4–775.1</td>
<td>6 67.0</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>6 460.9</td>
<td>77.6–1305.5</td>
<td>6 169.3</td>
<td>5.8–493.0</td>
<td>6 47.1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Profile of the mean concentrations of the main metabolites.
Experiments have been performed to study the suitability of the sample preparation conditions to separate the different metabolic fractions. It has been confirmed in this study that the cleavage of glucuronides subjected to sulphate fraction extraction was below the 6% level and Roig et al. have estimated that the hydrolysis efficiency with β-glucuronidase was over 90%. Another analytical approach has been described using methanolysis to cleave the glucuronide and sulphate conjugates in a single step to obtain the total fraction and using hydrolysis with β-glucuronidase to obtain the glucuronide fraction. Using this approach, it is assumed that the hydrolysis efficiency of the glucuronides is the same with both hydrolysis methods, but it was not demonstrated. Moreover, although satisfactory hydrolysis efficiencies were obtained with this approach for testosterone and epitestosterone, other authors have reported variable cleavage and rearrangements with methanolysis, and thus, in our opinion, the suitability of this approach was not sufficiently verified.

A comprehensive metabolic profile in urine after oral administration of a nutritional supplement of NOL is presented in our study. The dosage recommended by the manufacturer was used for the clinical study, and two capsules of NOL were administered to the healthy volunteers. The main metabolites detected were NA and NE in the glucuronide or sulphate fraction and ENA in the sulphate fraction. This is in accordance with conjugation patterns for hydroxyl groups in steroid metabolism: glucuronide conjugates are formed at the 3α-positions and sulphates are formed at both the 3α- and the 3β-positions; the hydroxyl group in 17β-position is also conjugated with glucuronic acid or sulphate. The prevalence of NA and NE in the glucuronide or sulphate fraction fluctuates in the first hours after administration, but, after 24 h, all the volunteers excreted more NA in the sulphate fraction than in the glucuronide fraction. Similar results were obtained by Tseng et al., although direct evaluation of the data is not possible because ratios instead of concentrations of the sulphate fraction were given in the last study. These results are not in accordance with a former study reporting that urinary NA formed after ND administration is exclusively conjugated with glucuronic acid.

The concentration-time profile of main metabolites shows an initial concentration peak between 2 and 4 h and a second peak between 8 and 16 h (Fig. 2). A profile with two concentration peaks was also obtained in plasma samples from the same volunteers; plasma concentrations of NE glucuronide increase from 8 to 24 h post-administration after an initial decline. This observation might suggest the existence of two different metabolic pathways with different rates of NE formation; this could be related to two different isoforms of 5β-reductase enzyme.

One of the main findings of our study is the presence of ENE in the sulphate fraction. Previous studies of the metabolism of norsteroids have not mentioned this metabolite. Other metabolites in addition to the administered compound were found at low concentrations. NOL and ND could be observed in the free and glucuronide-conjugated fractions in all volunteers, although in the free fraction they were found at very low concentrations. NONE was the main metabolite in the free fraction. These minor metabolites, as well as the parent compound, have not been described previously.

The cumulative amounts of the metabolites excreted during the whole collection period correspond to almost half of the dose administered, in accordance with recent excretion studies after oral administration of norsteroids. The main metabolites are still found in urine 96 h after administration.

**Table 5.** Percentage of the dose excreted of each metabolite in each fraction (n = 6) (FF: free fraction; GF: glucuronide fraction; SF: sulphate fraction)

<table>
<thead>
<tr>
<th></th>
<th>FF Mean (SD)</th>
<th>GF Mean (SD)</th>
<th>SF Mean (SD)</th>
<th>Total Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>0.005 (0.001)</td>
<td>10.4 (4.0)</td>
<td>14.3 (4.1)</td>
<td>24.6 (6.7)</td>
</tr>
<tr>
<td>NE</td>
<td>0.025 (0.010)</td>
<td>11.0 (6.2)</td>
<td>5.0 (4.6)</td>
<td>16.0 (9.7)</td>
</tr>
<tr>
<td>ENA</td>
<td>—</td>
<td>—</td>
<td>4.8 (1.5)</td>
<td>4.8 (1.5)</td>
</tr>
<tr>
<td>ENE</td>
<td>—</td>
<td>—</td>
<td>0.2 (0.1)</td>
<td>0.2 (0.1)</td>
</tr>
<tr>
<td>ND</td>
<td>0.002 (0.001)</td>
<td>0.1 (0.1)</td>
<td>—</td>
<td>0.1 (0.1)</td>
</tr>
<tr>
<td>NONE</td>
<td>0.049 (0.018)</td>
<td>—</td>
<td>—</td>
<td>0.05 (0.02)</td>
</tr>
<tr>
<td>NOL</td>
<td>0.006 (0.003)</td>
<td>0.03 (0.03)</td>
<td>—</td>
<td>0.04 (0.03)</td>
</tr>
</tbody>
</table>
administration, so the low urinary recovery can be explained taking into account the metabolites excreted over 96 h, and the presence of other metabolites or other excretion routes that need to be considered when a complete mass balance is performed. A previous study reported about 30% of the intramuscular ND dose being excreted as urinary metabolites, although the norsteroid and the method of administration were not the same. In our opinion the difference from our study is mainly due to the sulphate fraction that was not considered in the previous work.

After 24 h, all the volunteers excreted more NA in the sulphate fraction than in the glucuronide fraction and 58.1% of the total amount of NA detected in 96 h is excreted in the sulphate form. These results suggest that NA in the sulphate form should be taken into consideration when defining the positive criterion of 19-norsteroids consumption. According to current WADA guidelines the misuse of 19-norsteroids is detected by the presence of NA in urine at a concentration greater than 2 ng mL⁻¹, measured in the free plus glucuronide fractions. Our results suggest that NA sulphate determination would allow the detection of the consumption of norsteroids over a longer period of time. However, more studies should be performed to determine the metabolic profile after 96 h post-administration, when the concentrations of the metabolites are close to the established threshold concentration.

CONCLUSIONS

The metabolic profile in urine after oral administration of NOL is presented. The main metabolites detected were NA and NE in the glucuronide and sulphate fractions and ENA in the sulphate fraction. The relative abundances of NA and NE in the glucuronide and sulphate fractions fluctuate with time, although after the whole collection period NA sulphate is the main metabolite. Minor metabolites were also determined and the detection of ENE in the sulphate fraction has been reported here for the first time. Our results suggest that measurement of NA sulphate should be considered in antidoping control in order to increase the retrospectivity of administration of 19-norsteroids.

Acknowledgements

The authors are grateful for finance received from the Spanish (Ministerio de Educacio´ n y Ciencia; Consejo Superior de Deportes) and Catalan (Departament d’Universitats, Recerca i Societat de l’informacio; Consell Catala´ de l’Esport) authorities.

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47. DOI: 10.1002/rcm