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Atypical excretion profile and GC/C/IRMS findings may last for nine months after a single dose of nandrolone decanoate

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ABSTRACT

The use of the anabolic androgenic steroid nandrolone and its prohormones is prohibited in sport. A common route of nandrolone administration is intramuscular injections of a nandrolone ester. Here we have investigated the detection time of nandrolone and 19-norandrosterone and 19-noretiocholanolone metabolites in eleven healthy men after the administration of a 150 mg dose of nandrolone decanoate. The urinary concentrations of nandrolone and the metabolites were monitored by GC–MS/MS for nine months and in some samples the presence of 19-norandrosterone was confirmed by GC/C/IRMS analysis. The participants were genotyped for polymorphisms in PDE7B1 and UGT2B15 genes previously shown to influence the activation and inactivation of nandrolone decanoate. There were large inter-individual variations in the excretion rate of nandrolone and the metabolites, although not related to genetic variations in the UGT2B15 (rs1902023) and PDE7B1 (rs7774640) genes. After the administration, 19-norandrosterone was found at 2-8-fold higher concentrations than 19-noretiocholanolone. We showed that nandrolone doping can be identified 4 and 9 months after the injection of only one single dose in six and three individuals, respectively. We also noted that GC/C/IRMS confirms the presence of exogenous 19-norandrosterone in the urine samples, showing $\delta 13$ values around −32 ‰. This was true even in a sample that was not identified as an atypical finding after the GC–MS/MS analysis further showing the power of using GC/C/IRMS in routine anti-doping settings.

Nandrolone (17β-hydroxyestr-4-en-3-one) or 19-nortestosterone (19-NT) is one of the most abused anabolic-androgenic steroid (AAS) in sports (www.wada-ama.org) as well as in the society [1,2]. 19-Norandrosterone (19-NA; 3α-hydroxy-5α-estran-17-one) and 19-noretiocholanolone (19-NE; 3α-hydroxy-5β-estran-17-one) are the main metabolites of nandrolone. The presence of 19-NA in athletes' samples at a concentration above the Threshold (T) 2 ng/mL and more precisely above the decision limit (DL) at 2.5 ng/mL (adjusted to a specific gravity of 1.020) may result in an "Adverse Analytical Finding" (AAF) if an exogenous origin is proved [3].

A naturally minor production of 19-NA can be observed in women during pregnancy or at time of ovulation [4], after the consumption of boar meat [5], and possibly after administration of some progestagenic drugs like norethisterone [6]. In some very rare and particular conditions the *in situ* formation of 19-NA (via endogenous steroid 19-demethylation) in urine specimens stored for a long time has been described [7]. Since it is possible to detect 19-NA in true negative samples, a definitive assignment of the origin of 19-NA must be determined by the use of gas chromatography-combustion-isotope ratio mass spectrometry GC/C/IRMS [8]. According to WADA Technical Document-TD2015NA, GC/C/IRMS analysis shall be performed for any urine sample with 19-NA concentrations between DL and 10 ng/mL in men [3].

GC/C/IRMS is used to determine the $^{13}C/^{12}C$ ratio on the principle that natural steroids have a different carbon isotopic signature than synthetic 19-NT preparations. GC/C/IRMS has the potential to identify exogenous origin of 19-NA after the ingestion of norandrostenedione and norandrostenediol [8–10]. However, GC/C/IRMS analysis after an intramuscular injection of nandrolone decanoate (ND), the most common route for 19-NT administration, has not been assessed.

The urinary 19-NA in its glucuronidated form may be detectable up to one year after i.m injection of ND [11]. The excretion rate is subject to very large inter-individual variation [12,11,13] which may be due to genetic polymorphisms in steroid metabolizing

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enzyme genes. We have seen that a single nucleotide polymorphism (SNP) leading to an aspartic acid to tyrosine substitution in codon 85 (D85Y) in the Uridine 5'-diphospho-Glucuronosyl Transferases (UGT2B15) gene was associated with 19-NA glucuronidation activity in human liver microsomes [14]. Moreover, we have seen that two SNPs in the PDE7B1 gene determines the excretion rate of testosterone after administration of testosterone enanthate [15], and since PDE7B1 also is involved in the hydrolyses and activation of ND [16] it is possible that these SNPs also influence the urinary excretion rate of 19-NT and/or its metabolites.

Here we report the results of a study in healthy volunteers administered with 150 mg intramuscular ND in order to further increase our knowledge about pharmacokinetics of 19-NT in relation to detection time and different genotypes of the UGTs and PDE7B1 genes. Moreover, GC/C/IRMS analysis was conducted in order to study the usefulness of this technique to trace exogenous androgens long after intake.

1. Material and methods

1.1. Study population

Study subjects included 11 male volunteers' age 29–46 years (mean ± standard deviation 37.3 ± 5.0). All participants underwent a medical examination including laboratory tests before enrolment. They were all negative in screening tests for illegal drugs, AAS, HIV, hepatitis B or C virus. None was on any other medication or dietary supplements. The participants were given 150 mg ND as an intramuscular dose of Deca Durabolin® (Organon). Morning urine samples were collected prior to (day 0), and 1, 3, 5, 7, 14, 30, 60, 120 and 270 days after the administration. All participants gave informed consent consistent with the approval of the Ethics Review Board.

1.2. Chemicals

Potassium dihydrogen phosphate (KH2PO4), potassium hydrogen phosphate (K2HPO4), sodium sulfate (Na2SO4), potassium carbonate (K2CO3) and ethanethiol (C2H6S) were provided by Merck (Darmstadt, Germany). Ammonium iodide (NH4I) and methanol were provided by Sigma-Aldrich Chemie GmbH (Munich, Germany). n-Pentane was provided by Fisher Scientific (UK). The enzyme β-glucuronidase from *Escherichia coli* (*E. coli*) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). N-methyl-N-trimethylsilyl trifluoro acetamide (MSTFA) was provided by Macherey-Nagel GmbH (Düren, Germany). 19-Norandrosterone glucuronide (19-NAG), 19-noretiocholanolone glucuronide (19-NEG), 19-nortestosterone glucuronide (19-NTG) and Certified Reference material NMIA MX002, 19-NA in Freeze-Dried Human Urine at concentration close to the threshold of 2 ng/mL were purchased from NMI (Pymble, Australia). The following deuterated internal standards were obtained from NMI (Pymble, Australia): d4-19-norandrosterone glucuronide (d4-19-NAG), d4-19-noretiocholanolone (d4-19NE) and d3-epitestosterone (d3-epiT).

1.3. Sample preparation

The urine samples were analyzed and quantified for the presence of 19-NA, 19-NE and 19-NT using gas chromatography tandem mass spectrometry GC–MS/MS. Samples were stored at −20 °C awaiting analysis. The samples were divided in two groups based on expected concentration in urine. For samples collected on day 0, 120 and 270, 2 mL urine was used for the analysis. The concentrations of 19-NA, 19NE and 19-NT were calculated against a 5 points standard curve between 1 and 50 ng/mL. For samples collected on days 1, 3, 5, 7, 14 and 30, a volume of 0.5 mL of urine was used except for 1 mL for day 60. Standard curve included samples between 100 and 1000 ng/mL.

The urine samples were prepared according to an established nandrolone confirmation procedure in use in our laboratory. Mixtures of deuterated internal standards, d4-19NAG, d4-19-NE and d3-epiT were added to urine samples. The conjugated metabolites were hydrolyzed with 50 μL of β-glucuronidase enzyme from *E. coli* in 0.5 mL of 1 M potassium phosphate buffer pH 7.0 for 1 h at 50 °C. Following hydrolysis 250 μL 20% potassium carbonate solution was added and the steroids were extracted with 5 mL n-pentane at pH 9.6. The pentane extract was subsequently evaporated to dryness in nitrogen, samples were derivatised with 100 μL of MSTFA/NH4I/ethanethiol during 30 min. at 60 °C.

1.4. Urinary analysis – GC–MS/MS and GC/C/IRMS

Chromatographic separation was performed on a gas chromatography system Agilent 7890A equipped with a column Agilent HP-Ultra1 (17 m × 0.2 mm I.D., 0.11 μm) (Part nr 19091A-008). A constant helium flow of 1.2 mL/min was applied. The extracted sample (3 μL) was injected in the split mode (1:10). The temperatures were set at 250, 260, 150 and 230 °C for the injector, transfer line, quadrupole and ionization source, respectively. The GC oven temperature program was set as follows, start at 180 °C before gradient increase at 3.3 °C/min to 231 °C, finally 30 °C/min to 310 °C and maintained 2 min before the next analysis.

The Gas Chromatograph was coupled to the Agilent Triple Quadrupole Mass Spectrometer 7000C. The EI ionization was performed at 70 eV. The quantification was performed in the MRM mode (Table 1). Identity of 19-NA, 19-NE and 19-NT was controlled by the stability of the peak area ratios as described in [17]. The quantification was corrected by d4-19NA, d4-19NE and d3-epiT as internal standards respectively.

The GC/C/IRMS method was employed at the Doping Control Laboratory in Ghent, Belgium. A detailed description of the sample preparation procedure was published previously [18] and the full GC/C/IRMS method was described in a doctoral thesis [19]

Table 1
Agilent 7890A-7000C Triple Quadrupole GC–MS/MS system analysis parameters for nandrolone and the metabolites.

RT	Compound	Precursor ion	Product ion	Dwell time (ms)	Collision energy (eV)
7.83	19-NA	420	405	30	10
		420	315	30	20
		420	225	30	25
		405	315	30	10
		405	225	30	15
7.80	IS d4-19-NA	424	409	30	10
		409	319	30	10
		409	229	30	15
8.68	19-NE	420	405	19	10
		420	315	19	20
		420	225	19	25
		405	315	19	10
		405	225	19	15
8.63	IS d4-19NE	424	409	19	10
		409	319	19	10
10.76	19-NT	418	194	18	20
		418	403	18	15
		418	328	18	10
		418	313	18	20
		418	182	18	20
10.77	IS d3-epiT	435	330	17	25
		438	209	17	20