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A simplified procedure for GC/C/IRMS analysis of underivatized 19-norandrosterone in urine following HPLC purification

Xavier de la Torre[a,*], Cristiana Colamonici[a], Davide Curcio[a], Francesco Molaioni[a], Marta Pizzardi[a], Francesco Botrè[a,b]

[a] Laboratorio Antidoping, Federazione Medico Sportiva Italiana, Largo Giulio Onesti 1, 00197 Rome, Italy
[b] Dipartimento di Management e Tecnologie, 'Sapienza' Università di Roma, Via del Castro Laurenziano 9, 00161 Rome, Italy

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ABSTRACT

Nandrolone and/or its precursors are included in the World Anti-doping Agency (WADA) list of forbidden substances and methods and as such their use is banned in sport. 19-Norandrosterone (19-NA) the main metabolite of these compounds can also be produced endogenously. The need to establish the origin of 19-NA in human urine samples obliges the antidoping laboratories to use isotope ratio mass spectrometry (IRMS) coupled to gas chromatography (GC/C/IRMS). In this work a simple liquid chromatographic method without any additional derivatization step is proposed, allowing to drastically simplify the urine pretreatment procedure, leading to extracts free of interferences permitting precise and accurate IRMS analysis. The purity of the extracts was verified by parallel analysis by gas chromatography coupled to mass spectrometry with GC conditions identical to those of the GC/C/IRMS assay. The method has been validated according to ISO17025 requirements (within assay precision of ±0.3‰ and between assay precision of ±0.4‰). The method has been tested with samples obtained after the administration of synthetic 19-norandrostenediol and samples collected during pregnancy where 19-NA is known to be produced endogenously. Twelve drugs and synthetic standards able to produce through metabolism 19-NA have shown to present $\delta^{13}C$ values around −29‰ being quite homogeneous (−28.8 ± 1.5; mean ± standard deviation) while endogenously produced 19-NA has shown values comparable to other endogenous produced steroids in the range −21 to −24‰ as already reported. The efficacy of the method was tested on real samples from routine antidoping analyses.

1. Introduction

19-Norandosterone (19-NA) is in humans the main metabolite of some anabolic androgenic steroids (AAS) like 19-nortestosterone (nandrolone), 19-norandostenedione and 19-norandostenediol [1–5], but can be also detected as a minor metabolite of norethandrolone or ethylestrenol [6]. The use of AAS is prohibited in sports and these compounds are included in the prohibited list of forbidden compounds and methods published and periodically updated by the World Anti-Doping Agency (WADA) [7].

The detection of 19-NA is also possible after the administration of some progestagenic drugs as norethisterone [8,9].

In addition to the previously mentioned synthetic origin, the naturally occurring production of 19-NA has been demonstrated in animals and human males [10–15]. In females, due to the production of large amounts of estrogens during pregnancy or ovulation, 19-NA can be produced as a by-product of aromatization [16,17] leading to detectable concentrations of 19-NA in urine by the routine mass spectrometric procedures applied by WADA Accredited Laboratories (GC/MS, GC/MS/MS or GC/HRMS). In males this metabolic route is less expressed leading to the production of trace amounts of 19-NA [18]. A cut-off value of 2 ng/mL (to be adjusted as a function of the specific gravity of the sample and considering the uncertainty of the method) has been established by the international antidoping authorities to disclose from the synthetic to the natural origin of 19-NA in urine of sportsmen [19]. In some very rare and particular conditions the in situ formation of 19-NA (by endogenous steroids 19-demethylation) in urine specimens after a long term storage of the samples has been described [20]. Finally it has been reported, even if extremely improbable, that the consumption of meat (i.e. from non-castrated pigs or boars) can result in the later excretion of nandrolone metabolites into urine since 19-NA is endogenous in some species [21,22]. As a consequence, for a definitive assignment of the 19-NA origin finding in urine the use of GC/C/IRMS has become a requirement for WADA accredited laboratories [23], and specifically for those samples with low 19-NA concentrations (<10 ng/mL). The analysis by GC/C/IRMS requires

* Corresponding author. Tel.: +39 06 36859600; fax: +39 06 8078971.
E-mail address: xavier.delatorre@gmail.com (X. de la Torre).

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Table 1
Characteristics of published methods for the analysis of 19-NA and related compounds by GC/C/IRMS.

#	Sample volume (mL)	Sample pre-concentration	Purification		Derivatization	LOD (ng/mL)	Ref.
			Step 1	Step 2			
1	40	SPE (2×)	IAC	None	None/acetylation	30–40	[24]
2	20–50	SPE + L/L	HPLC (Inertsil NH2)	HPLC (Inertsil NH2)	Acetylation	25	[27][a]
3	20	SPE (C18)	SPE (silica-gel)	SPE (C18)	Acetylation	30	[25]
4	10	SPE + L/L	HPLC (dimethylaminopropyl)	HPLC (C18)	None	2	[26]

IAC: immuno affinity chromatography; SPE (solid phase extraction); L/L: liquid/liquid extraction.
[a] Analysis of 5α-estrane-3β,17α-diol in horse urine.

an adequate sample pretreatment to avoid potential interferences. Different exhaustive approaches for sample pretreatment for 19-NA based on immunoaffinity chromatography (IAC) [24], combined solid phase extraction [25] or combined double liquid chromatography [26,27] have been published. Furthermore, to improve the thermal stability and to increase the volatility of the target compounds, as to improve their GC profile, chemical derivatization on the preconcentrated and purified urine extracts is often performed, resulting in an additional step prior to the GC/C/IRMS analysis (Table 1).

The aim of this work is to develop a simple method for sample pretreatment for 19-NA by liquid chromatography prior to its analysis by GC/C/IRMS, with no need of chemical derivatization.

2. Experimental

2.1. Standards and reagents

The standards of androsterone (A; 3α-hydroxy-5α-androstane-17-one), 19-norandrosterone (19-NA; 3α-hydroxy-5α-estrane-17-one), and 19-NA-3-O-glucuronide were purchased from NMIA (Pymble, Australia), 5α-androstan-3β-ol was from Steraloids (Newport, RI, USA) and 17α-methyltestosterone (MT) was from Sigma–Aldrich (Milano, Italy).

All reagents and solvents (sodium bicarbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate, *tert*-butylmethylether, acetonitrile, methanol, n-pentane, cyclohexane and isopropanol) were of analytical or HPLC grade and provided by Carlo Erba (Milano, Italy). β-Glucuronidase from *Escherichia coli* K12 was from Roche Diagnostic (Mannheim, Germany). Water was from a Milli Q water purification system (Millipore S.p.A, Milano, Italy).

CO_2 reference gas (Solgas, Monza, Italy) for isotope ratio mass spectrometer calibration was calibrated against underivatized steroids (CU/USADA34-1) with certified delta values traceable to VPDB, obtained from Prof. Brenna (Cornell University Ithaca, NY) [28].

2.2. Urine samples

For the validation of the method, samples obtained after the consumption of synthetic nandrolone precursor and of 19-NA produced endogenously were collected. In addition samples founded to be positive for the presence of 19-NA during routine doping control analysis were analyzed.

2.2.1. Excretion study with norandrostenediol

A healthy male volunteer (41 yr, 77 kg weight) (after an informed consent approval) was administered orally with a single capsule of the prohormone dietary supplement Norandrodiol Select 300 (Ergopharm, Champaign, IL, USA) containing 150 mg of norandrostenediol (19-norandrost-5-en-3β,17β-diol). A blank urine sample was collected before and spot samples were collected after the administration for the next 9 days. Samples were stored at −20 °C until analysis. The composition and $\delta^{13}C$ content were analyzed before the administration excluding the presence of other compounds in the formulation (see Section 2.4 for details).

2.2.2. Samples under special physiological conditions

Urine samples from a pregnant woman (33 yr, 44 kg weight) were collected from the 12th week of pregnancy until delivery.

Additionally daily spot urine samples produced during two menstrual cycles (24 yr, 66 kg weight; 26 yr, 60 kg weight) were collected.

In these samples gonadotrophines (luteinizing hormone, LH; follicle stimulating hormone, FSH and human chorionic gonadotrophin, hCG) were measured by chemioluminiscence (Siemens Immulite® 2000 DPC, Medical Systems SpA, Genoa, Italy) using the protocol recommended by the manufacturer. In addition the concentrations of estrone (E1) and estradiol (E2) were measured by GC/MS by the application of the method previously described [29]. Samples were collected in sterile containers and stored at −20 °C until analysis.

2.3. Sample preparation

Urine samples (21 mL, 3× 7 mL) were extracted with 10 mL of tertbutylmethyl ether after the addition of 1 mL of phosphate buffer (0.8 M, pH 7) to separate the free from the conjugated fraction. Once the free fraction discarded, the hydrolysis was initiated by the addition of 50 μL of β-glucuronidase from *E. coli*. The hydrolysis was performed at 55 °C during 60 min; after cooling, pH was adjusted to 9–10 with carbonate buffer (20%) and extraction performed with 10 mL of n-pentane. Once the solvent separated the 3 aliquots were combined and taken to dryness, then the final residue was dissolved in 50 μL of a mixture water/methanol (50/50) containing methyltestosterone (100 μg/mL) for subsequent HPLC purification of the extract.

2.3.1. HPLC sample purification

Sample purification was performed using an Ascentis phenyl column from Supelco (Sigma–Aldrich, Milano, Italy) (15 cm, 4.6 mm, 3 μm) at 60 °C. Separation was programmed on an Agilent 1100 Series liquid chromatograph (Agilent Technologies SpA, Cernusco sul Naviglio, MI, Italy) with a mobile phase composed with water (solvent A) and acetonitrile (solvent B). For compounds separation, an isocratic program was set up set up at 50% B for 8 min then increasing to 100% B in 0.01 min. The column was flushed for 7 min at 100% B and finally re-equilibrated at 50% B for 5 min for a total run time of 20 min. The flow rate was set at 1 mL/min. Separation conditions were established by monitoring the signal of a UV lamp at 192 nm (Agilent 1100 UV DAD detector). All the volume of sample extract obtained was injected in the HPLC/UV system. Methyltestosterone (MT) was used to control the reproducibility of the elution conditions and based on this, elution fractions around the expected retention times of 19-NA (6.3 min) and of androsterone (7.2 min) were collected on a programmed Agilent 1100 fraction collector. Fractions before and after the fractions contain-