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Norandrosterone analysis by GC/C/IRMS

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Abstract

Since nandrolone's metabolites, 19-norandrosterone (5α-estran-3α-ol-17-one, 19NA) and 19-noretiocholanolone (5β-estran-3α-ol-17-one, 19NE) are known to be also produced endogenously in the human body, the use of GC/C/IRMS (gas chromatography/combustion/isotope ratio mass spectrometry) has become a necessity to detect its abuse in sport. The WADA technical document requests the comparison between $\delta^{13}C$ values of 19NA to $\delta^{13}C$ of an endogenous reference compound (ERC) for samples showing 19NA concentrations higher than 2 ng/mL in urine.
A newly developed method will be presented herein allowing for the detection of 19NA, 19NE, androsterone (5α-androstan-3α-ol-17-one, A), pregnanediol (5β-pregnan-3α,20α-diol, PD) and 16-androstenol (16,(5α)-androsten-3α-ol, 16EN) by GC/C/IRMS.
The usual purification steps (solid phase and liquid-liquid extraction, hydrolysis and twofold HPLC purifications) were maintained for sample preparation. However, modifications in the twofold HPLC purification and an additional acetylation step were found to be necessary to improve determination of 19NA and 19NE. The first HPLC purification was performed on a Waters XBridge™ Shield RP18 column allowing for the complete separation of the ERCs (A, PD and 16EN). Only the fractions containing 19NA and 19NE did necessitate a second HPLC purification with a Waters XBridge™ RP18 column after acetylation to ensure the purity of compounds.
The method was validated and did already demonstrate its benefit to detect the exogenous intake of nandrolone or its precursors in anti-doping samples over the last year.

Introduction

As 19-norandrosterone (5α-estran-3α-ol-17-one, 19NA) and 19-noretiocholanolone (5β-estran-3α-ol-17-one, 19NE) can be produced endogenously in the human body, the use of GC/C/IRMS (gas chromatography/combustion/isotope ratio mass spectrometry) became a necessity to detect nandrolone (17β-Hydroxyestra-4-en-3-one) abuse in sport [1]. In conclusion, the World Anti-Doping Agency (WADA) request the comparison of $\delta^{13}C$-values of 19NA to an endogenous reference compound (ERC) for 19NA concentrations higher than 2 ng/mL in urine for the glucuronidated compound [2]. A newly developed method will be presented herein allowing simultaneous determination of 19NA, 19NE, androsterone (5α-androstan-3α-ol-17-one, A), pregnanediol (5β-pregnan-3α,20α-diol, PD) and 16-androstenol (16,(5α)-androsten-3α-ol, 16EN) by GC/C/IRMS from one urine aliquot.

Experimental

HPLC clean up
Processing of samples encompassed the usual steps [1,3,4] followed by two HPLC runs. The first fractionation step was performed on an Agilent 1100 HPLC system (Waldbronn, Germany) with a Xbridge™ Shield RP18 5 mm (4.6 x 250 mm) column protected with a XBridge™ Shield RP18 5 mm (4.6 x 20 mm) guard column purchased from Waters (Baden-Dättwil, Switzerland). The injection volume was 50 µL and the flow rate 1 mL/min. A linear gradient was used increasing from 40/60 ACN/H_2O to 60 % ACN in 18 min and then within 1 min to 98 % ACN. After 11 min at 98 %, the column was re-equilibrated for 10 min. Five fractions were collected: 19NE (12.2 – 13.5 min), 19NA (14.5 – 15.8 min), A (15.8 – 17.4 min), PD (19.8 – 21.2 min) and 16EN (22.5 – 23.8 min).

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After acetylation, fractions containing 19NA and 19NE underwent a second clean up on the same HPLC system now using a Xbridge™ RP18 5 mm (4.6 x 150 mm) column. The gradient was changed to start at 60/40 ACN/H_2O, increased to 98 % ACN in 16 min, was hold for 9 min and subsequent the column was re-equilibrated as above. Both target analytes (19NA_Ac and 19NE_Ac) were collected and forwarded to IRMS measurements.

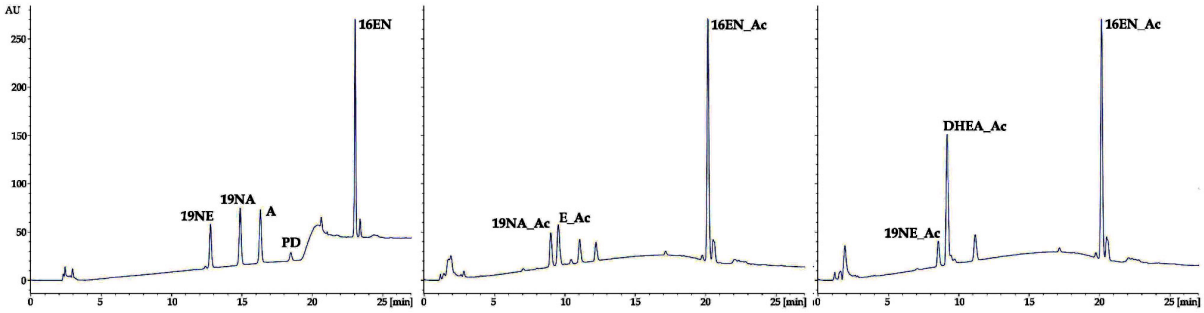

Figure 1: UV-chromatograms (wavelength 195 nm) of standards containing the relevant steroids.

GC/C/IRMS measurements
All samples were measured on an Agilent 7890 gas chromatograph (Waldbronn, Germany) coupled to a Delta V gas isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) via the GC combustion interface (GCC III, Thermo Electron). A detailed description of the method has already been published [4].

Method validation
The method has been tested and validated by fourfold sample preparation of six different blank urines fortified with 5, 10 and 20 ng/mL 19NA and 19NE. One urine remained blank to test for specificity of the method.

Results and Discussion

Method validation
No significant influence on isotopic ratios was visible caused by any step of sample preparation as shown in Table 1. The standard deviation for 19NE was found slightly elevated in contrast to 19NA due to an endogenous substance co-eluting in some of the urines tested during validation. Nevertheless, the mean values for standard measurements and spiked urine were found equal.

	Std_19NA	Std_19NE	QC_19NA	QC_19NE	QC_PD
mean	-26.90	-29.39	-27.30	-28.83	-22.81
SD	0.15	0.15	0.74	1.15	0.44

Table 1: Results obtained for standards (n = 6) and spiked urines (n = 18). All values given in $\delta^{13}C_{VPDB}$ [‰].

GC/C/IRMS measurements
In Figure 2 the benefit of the extensive sample preparation is depicted. For 19NA the chromatograms for a standard (upper part) and a doping control sample (lower part) look nearly similar. For 19NE a quality control urine (10 mL processed) fortified at 10 ng/mL and for 19 NA a doping control sample (15 mL processed) with 4.4 ng/mL are presented. As mentioned, a small co-elution is detectable with 19NE. Additionally, the same absolute amount of 19NE injected on the IRMS system always results in lower signal intensity compared to that for 19NA. An interesting finding that we cannot explain unambiguously at the moment which might also contribute to the larger standard deviation for 19NE.

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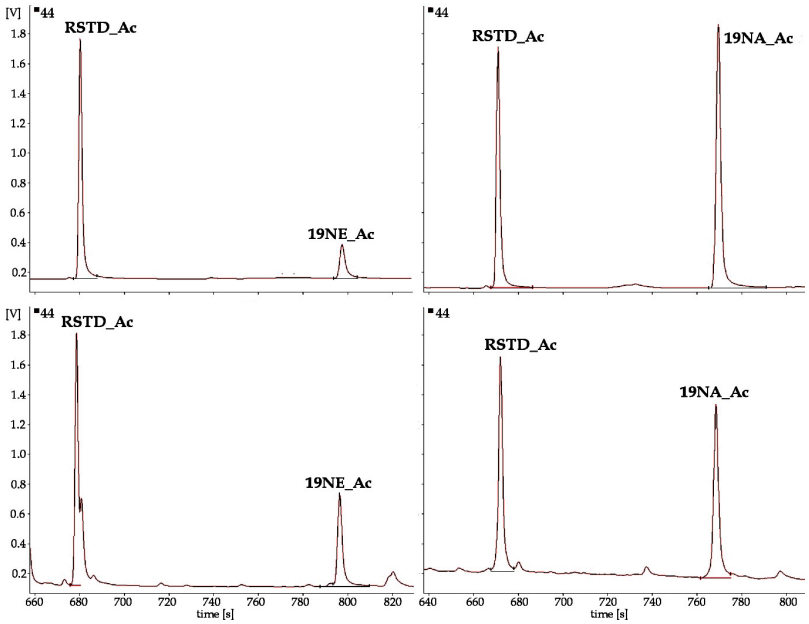

Figure 2: GC/C/IRMS chromatograms of standards (upper part) and samples (lower part).

The method for 19NA is regularly used for doping control samples. Table 2 summarizes $\delta^{13}C$-values obtained for two samples collected from one athlete. A- and B-sample analysis were performed on both specimens and the obtained values were found in perfect agreement. The Δ-value for PD-19NA was -6.5 ± 0.49 ‰ for all four samples investigated over a time period of two months. With this standard deviation the method meets the current WADA requirements.

	PD	A	19NA
First_A	-21.2	-21.0	-27.3
First_B	-21.1	-21.1	-28.4
Second_A	-21.9	-21.5	-28.4
Second_B	-21.6	-20.8	-27.8
mean	-21.4	-21.1	-28.0
SD	0.39	0.30	0.50

Table 2: Results for A and B sample analysis for two doping control samples from the same athletes. All values given in $\delta^{13}C_{VPDB}$ [‰].

Conclusions

The developed method enables valid measurements of 19NA and fulfills the associated WADA requirements [2]. The first attempts to measure also 19NE were promising and after adjusting the HPLC parameters it should be possible to determine this nandrolone metabolite, too. In the near future this new method will be tested for its suitability to determine also 19NA and 19NE excreted as sulfates into urine. This might become interesting for doping control analysis as the urinary concentration of 19NE sulfate is often found elevated if the administration of nandrolone dates back a long time.

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