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Nandrolone criteria for 19-norandrosterone Isotope Ratio Mass Spectrometric confirmation

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Abstract

The scientific rationale for the use of isotope ratio mass spectrometry (IRMS) as a confirmatory technique of the abuse of pseudoendogenous steroids is the different content of ^{13}C and ^{12}C in the synthetic preparations compared to the endogenously produced steroids.
According to the WADA Technical Document (TD2012NA), to reject the hypothesis of endogenous 19-NA formation, based on the application of GC/C/IRMS analysis, the $\delta\ ^{13}C$ value of 19-NA must be outside the range of values normally measured in humans (i.e. is less than -27 ‰) and simultaneously the Δδ value between the endogenous reference compound (ERC) (e.g. androsterone (A) or pregnanediol (PD)) and 19-NA, i.e. $\Delta\delta = \delta_{ERC} - \delta_{19\text{-}NA}$, is greater than 3 ‰.
Since the normal population, with some differences depending on the dietary habits, shows values in the range -19 to -26 ‰, any synthetic preparation in the range -23 to -26 ‰ would greatly invalidate this approach. The presence of preparations of pseudoendogenous compounds, from illicit market, showing such delta values have been detected and reported.
We report some cases suggesting the presence in the market of nandrolone formulations not detectable with the current WADA criteria. A revision of these criteria, following the same approach adopted for the confirmation of testosterone precursors, would be necessary.

Introduction

The detection of the use of nandrolone and other 19-norsteroids is based primarily upon the identification of the main urinary metabolite, 19-norandrosterone (19-NA) in a concentration greater than the Decision Limit (DL), as established in the DL Technical Document. The presence of 19-NA in urine samples can be originated by *in-vivo* endogenous production (ovulation or pregnancy in females) and/or exogenous administrations (nandrolone or nandrolone precursors) or by *ex-vivo* production in urine by in-situ 19-demethylation of androsterone (A) (active urines).
The use of isotope ratio mass spectrometry (IRMS) for the unambiguous determination of the endogenous or synthetic origin of 19-NA is in some circumstances mandatory to report an adverse analytical finding (AAF). In this work, we review the applicability of the current criteria (TD2012NA) on real samples.

Experimental

The samples object of this investigation were collected during the routine Antidoping program in India and once the suspicious samples for 19-NA were quantified by GC/MS, they were submitted for GC/C/IRMS confirmation.

GC/MS analysis
Two mL of sample were hydrolyzed with 30 µL of enzyme (*E. coli*) for 1h at 55°C after the addition of the internal standard (19-NA d_4 at a final concentration of 2 ng/mL) and 750 µL of phosphate buffer (0.8 M). The free and deconjugated steroids were then extracted with 5 mL of *n*-pentane at pH 9-10 after 250 µL addition of Na_2CO_3/$NaHCO_3$ (20%). The organic solvent

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was taken to dryness and the final residue dissolved in 50 µL of MSTFA/NH_4I/2-mercaptoethanol (1000:2:6). Two µL were injected in a GC/MS/MS instrument (Agilent 7890A gas chromatograph coupled to a 7000B triple quadrupole mass spectrometer) in SRM acquisition mode for the identification and quantification analysis. A 5 points calibration curve was built up by adding known amounts of 19-NA to 2 mL of blank urine at the final concentrations of 1, 2, 5, 10 and 15 ng/mL, respectively. The calibration curve followed the same sample preparation as the real samples.

IRMS
For the 19-NA GC/C/IRMS confirmation analysis, the validated method routinely used in our laboratory and described in [2] starting from 21 mL of urine was applied. When possible, multiple endogenous reference compounds (ERCs) as androsterone (A), 11-ketoetiocholanolone (11keto) and pregnantriol (P3) were monitored.

Data analysis
The analytical data (quantified nandrolone concentrations, the observed absolute $\delta^{13}C$ delta values and the Δδ ratios) were imported into SIMCA-P+ 12 (Umetrics, Umea, Sweden) for statistical data analysis.

Results and Discussion

From the literature data [2,3] for the formulations containing 19-NA precursors and the in-house reference population for an ERC (A), two distinct populations are depicted (Figure 1), demonstrating the usefulness of IRMS to detect exogenous 19-NA.

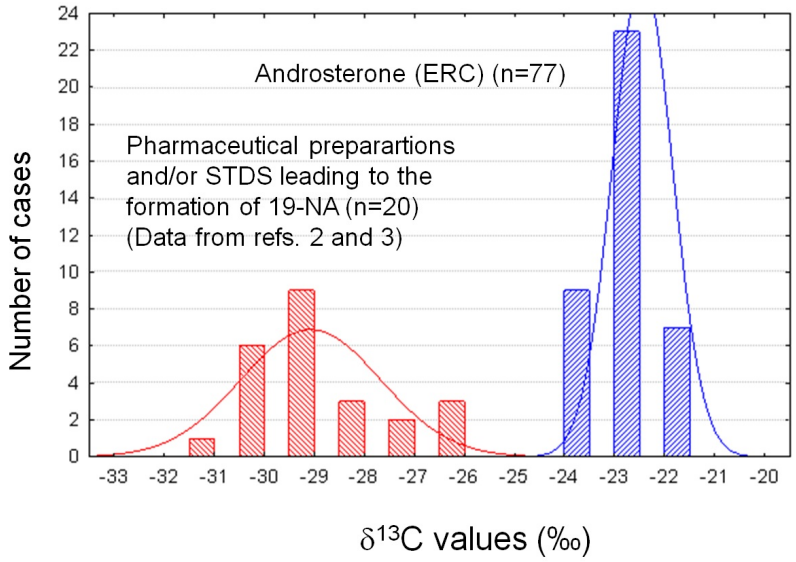

Figure 1. Distribution of the ^{13}C delta values of 19-NA precursors and ERC (A)

However, a critical area in the range -23 to -26 ‰ may create some problems for the correct interpretation of the cases.
In Table 1 the analytical findings obtained from 12 cases studied are summarized. Results of cases 2B, 3B and 4B corresponding to the B samples of cases 2, 3 and 4 demonstrate the good repeatability of the IRMS method applied.
From the 12 cases, 9 fulfill all the WADA criteria to release an AAF, while the other 3 are more problematic. Case #1 although showing a Δδ value > 3 ‰, the absolute 19-NA delta value was on the “endogenous” region (-25‰). Cases #10 and 11 showed Δδ values that are compatible with an endogenous production but absolute delta values were similar to case #1. The analysis did not demonstrated the presence of 2 statistical distinct populations. However the two populations clearly observed need to be confirmed by increasing the number of data. Case #10 belonged to a female athlete, and pregnancy or the use or progestins were excluded according to the WADA TD2012NA document. There are not reported cases of such high concentration of 19-NA compatible with the ovulation period.

Compared to the reference Caucasian population shown in Figure 1, most of the ERC (A) of the samples collected in India are on the upper part of the distribution (-21.9±0.9‰), increasing remarkably the possibility of having samples with a Δδ >3‰ with absolute values for 19-NA below -27‰. We cannot excluded that nandrolone precursors preparations, with delta values close to the endogenous region, are present in the market as has been detected and reported for testosterone [4].

Case #	Sport	Sex	Conc.	$\delta^{13}C$	ERC $\delta^{13}C$			$\Delta\delta$ (A - 19-NA)	Conclusion (*)
			19-NA	19-NA	A	11keto	P3		
			(ng/mL)	(‰)	(‰)				
1	ATHLETICS	M	5.8	-25,0	-20,9			4,1	Negative
2	BASKETBALL	M	9.8	-30,1	-21,9	-21,3	-22,3	8,2	Positive
3	KABADDI	M	6.8	-31,2	-21,7	---	-22,1	9,5	Positive
4	WEIGHTLIFTING	M	5.8	-29,5	-21,8	-22,3	-22,7	7,7	Positive
5	WEIGHTLIFTING	M	6.5	-29,7	-23,1	-22,8	-23,7	6,6	Positive
6	ATHLETICS	M	8.6	-30,5	-24,5	-23,2	-24,7	6,0	Positive
2B	BASKETBALL	M	9.8	-30,4	-21,8	-21,6	-21,9	8,6	Positive
7	HANDBALL	M	4.3	-29,3	-21,9	-22,0	-22,3	7,4	Positive
8	HANDBALL	M	7.2	-30,4	-21,8	-21,6	-22,6	8,6	Positive
3B	KABADDI	M	6.8	-31,1	-21,6	---	-22,0	9,5	Positive
4B	WEIGHTLIFTING	M	9.4	-29,4	-21,9	-21,9	-22,9	7,5	Positive
9	ATHLETICS	M	8.7	-29,9	-21,9	-21,5	-22,9	8,0	Positive
10	ATHLETICS	F	8.3	-24,2	-21,9	-21,7	-22,3	2,3	Negative
11	CYCLING	M	9.8	-25,0	-22,3	-22,5	-23,6	2,7	Negative
12	TABLE TENNIS	M	3.9	-31,4	-20,2	-20,3	-20,8	11,1	Positive

Table 1. Summary of the analytical data obtained in 12 samples showing elevated 19-NA concentrations

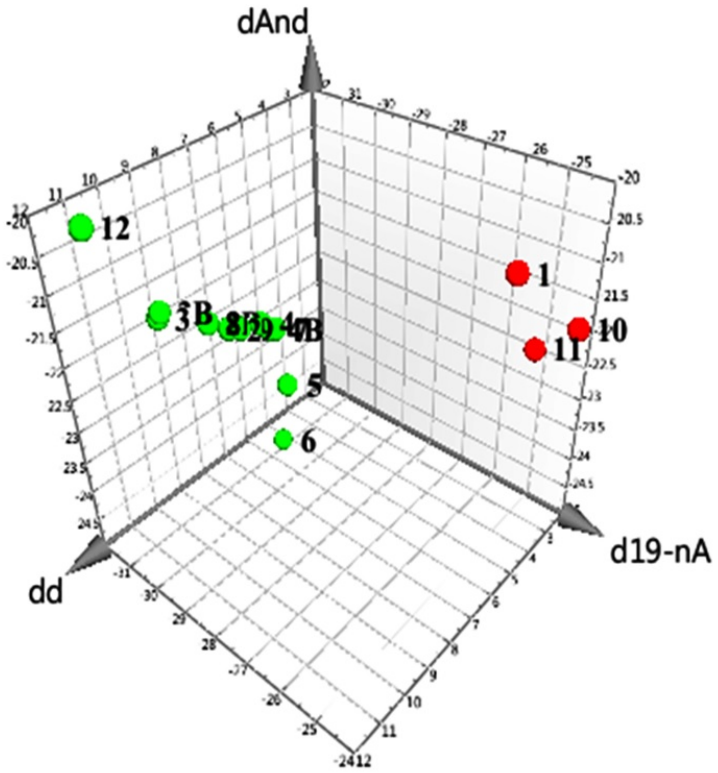

Figure 2. 3D graphical representation showing the presence of two distinct populations of samples (the three dimensions are $\delta^{13}C$ A (dAnd); $\delta^{13}C$ 19-NA (d19-nA) and the $\Delta\delta_{(ERC - 19\text{-}NA)}$ (dd)).

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Conclusions

In order to keep the validity of the IRMS confirmation analysis, a continuous survey of the delta values of potential nandrolone precursors present in the market is needed. Based on the current data, a revision of the current criteria to release an AAF for 19-NA is advisable considering what has been suggested for the detection of synthetic forms of endogenous anabolic androgenic steroids by IRMS (i.e. testosterone or its precursors), in the new WADA technical document, with the inclusion of the inconclusive cases when not all criteria are met.

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