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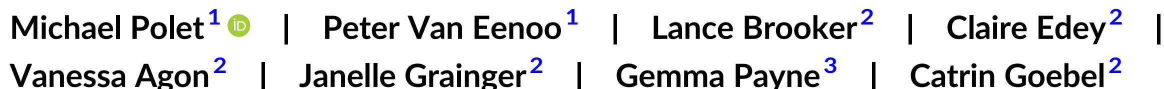
Prevalence of nandrolone preparations with endogenous carbon isotope ratios in Australia



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Abstract

Nandrolone and its prohormones, including 19-norandrost-4-ene-3,17-dione and 19-norandrost-4-ene-3β,17β-diol, are anabolic steroids forbidden at all times in sports according to the World Anti-Doping Code Prohibited List and its metabolite 19-norandrosterone (19NA) is the preferred urinary target compound to identify their abuse. In recent years, an increasing number of 19NA isotope ratio mass spectrometry (IRMS) cases have arisen that, based on the initial testing procedure, were likely to result in an adverse analytical finding but were concluded negative after IRMS analysis. The current study was therefore set up to gain a better insight on the prevalence of nandrolone preparations with endogenous carbon isotope ratio values in Australia. Suitable workplace (non-athlete) urine samples that had previously been reported positive for 19NA were identified and analysed on IRMS. A total of 82% of the samples that were analysed were reported with enriched carbon isotope ratios of 19NA (i.e., 19NA greater than −26‰). This indicates that there is a high prevalence of nandrolone-containing anabolic androgenic steroid preparations in Australia that have 'endogenous' carbon isotope ratios which reduces the ability to identify exogenous nandrolone.

KEYWORDS
anti-doping, IRMS, nandrolone, steroid preparation

1 | INTRODUCTION

Nandrolone is included in the World Anti-Doping Code Prohibited List of substances with 19-norandrosterone (19NA, main metabolite) and 19-noretiocholanolone (19NE, minor metabolite) as the preferred urinary target compounds to identify its abuse.[1] Despite its potential exogenous origin 19NA can also be produced endogenously at low concentrations and at levels between 2.5 and 15 ng/mL, isotope ratio mass spectrometry (IRMS) is required to establish the exogenous origin of urinary 19NA.[2] Here, the carbon isotope ratio ($\delta^{13}C$) value of 19NA is compared with the $\delta^{13}C$ value of an endogenous reference compound (ERC) such as pregnanediol (PD) or 11-oxoetiocholanolone (11oxoEt).

In 2011, seven pharmaceutical/nutritional preparations, leading to the urinary excretion of 19NA after intake, were analysed with gas chromatography combustion IRMS (GC-C-IRMS) for carbon isotope ratio determination ($\delta^{13}C = -30.9‰$ to $-29.1‰$).[3] In work conducted at the Australian Sports Drug Testing Laboratory (ASDTL) in 2012, 9 veterinary preparations and 36 preparations seized by law enforcement (Australia $n = 18$, United States $n = 3$, Germany $n = 7$, Belgium $n = 8$) containing nandrolone showed $\delta^{13}C$ values of $-33.0‰$ to $-27.2‰$ and $-32.6‰$ to $-29.0‰$, respectively.[4] Following, in Norway in 2014, 15 seized nandrolone preparations showed $\delta^{13}C$ values in the range of $-31.5‰$ to $-26.7‰$.[5] In all these studies, all nandrolone preparations showed $\delta^{13}C$ values well in the exogenous range ($\delta^{13}C < -26.7‰$) and, based on the data provided at that

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time, synthetic preparations with $\delta^{13}C$ values in the endogenous range were likely to be rare to non-existent. On top of that, in the case of testosterone IRMS analyses, the carbon isotope ratio (CIR) of testosterone will, at a given time point, be determined by a mix of the administered exogenous testosterone and the endogenous testosterone that is already present, leading to dilution of the exogenous CIR with endogenous CIR values.[6] In the case of 19NA, there is most often no or very little endogenous contribution, meaning that 19NA IRMS analyses leading to adverse analytical findings (AAFs) were for many years clear cases with the $\Delta\delta^{13}C$ between 19NA and ERC well above the WADA 3‰ threshold. As explained above, 19NA CIR values were well in the exogenous range ($\delta^{13}C < -26.7$‰) and, in most countries, the CIR of the ERC is >−23‰.[7]

However, in recent years, more and more 19NA IRMS cases have arisen in anti-doping that, based on the initial testing procedure outcome (e.g., AAF for one or more other anabolic steroids, relatively high 19NA concentration; endogenous 19NA > 2 ng/mL is in general very rare) were likely to result in a 19NA IRMS AAF but have turned out negative.

In a particularly suspicious sample (19NA = 4.7 ng/mL, AAF for other exogenous anabolic steroids), the $\Delta\delta^{13}C$ only barely exceeded the WADA 3‰ threshold ($\Delta\delta^{13}C$ [PD − 19NA] = 3.2‰; $\Delta\delta^{13}C$ [11oxoEt − 19NA] = 3.4‰; $\delta^{13}C$ [19NA] = −24.9‰). A number of similar cases where the detected 19NA was very likely to be the result of doping abuse, but resulted in a negative finding after 19NA IRMS analysis, presented itself in our and in other doping control laboratories. This indicated that there were nandrolone preparations with endogenous $\delta^{13}C$ values appearing. Work conducted in 2018 confirmed that this was indeed the case.[8] Here, nine nandrolone preparations were examined, four of which had $\delta^{13}C$ values in the endogenous range ($\delta^{13}C$ values −22.5‰ to −21.5‰). In light of these recent developments, the current study was set up, aimed at determining the prevalence of nandrolone preparations with endogenous carbon isotope ratios in Australia.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

$NaH_2PO_4{\cdot}H_2O$, $Na_2HPO_4{\cdot}2H_2O$, $NaSO_4$ and toluene were purchased from Merck (Darmstadt, Germany); $NaHCO_3$, K_2CO_3 and methanol (CH_3OH) from Fisher Scientific (Leicestershire, UK). Acetonitrile, n-hexane, methyl tertbutyl ether (MTBE) and liquid chromatography (LC)–MS grade water were purchased from Biosolve (Valkenswaard, The Netherlands); β-glucuronidase from *Escherichia coli* K12 from Roche Diagnostics (Mannheim, Germany); carbon dioxide, helium, nitrogen and oxygen from Air Liquide (Bornem, Belgium).

2.2 | Collection of samples

Suitable workplace (non-athlete) urine samples from the period 2018–2021 that had previously been reported positive by the ASDTL for 19NA were identified, totalling 40 samples. The GC–MS/MS method that was used for this has been described in detail in previous work.[9] The majority of the samples were also positive for other prohibited substances. A total of 11 of the 40 samples were delivered to the laboratory with low urine volumes (<40 mL). Consequently, 12 samples did not have sufficient urine volume remaining for IRMS analysis, leaving 28 samples to be de-identified and shipped to DoCoLab (Ghent University) where the 19NA IRMS analyses were conducted.

A blind quality control sample was also delivered together with the 28 samples mentioned above. The certified urine NMI MX017 (certified 19NA $\delta^{13}C = -29.82$‰) was used for this purpose (National Measurement Institute, Australia).

2.3 | IRMS analysis

IRMS analyses were conducted according to a previously published method.[10] Urine aliquots (max 25 mL) were centrifuged for 5 min at 2000–2800 rpm. Before loading the sample onto the solid phase extraction (SPE) cartridge (Bond Elut C18, 500 mg, 3 mL, Agilent Technologies), two conditioning steps (2 mL CH_3OH, 2 mL H_2O) were done. After loading, a washing step was performed (2 mL 10% CH_3OH/H_2O), and the compounds were eluted with 4 mL CH_3OH. The methanolic extract was evaporated to dryness under nitrogen at 60°C, reconstituted in 1 mL of a 0.1 M pH 7 phosphate buffer and vortexed. A total of 50 μL of the β-glucuronidase enzyme was added and the sample was hydrolysed at 56°C in an oven for 60 min. Afterwards, the sample was cooled to room temperature, and 1 mL $NaHCO_3/K_2CO_3$ buffer (pH 9.5) and 5 mL of MTBE were added. Liquid–liquid extraction was performed by rolling for 20 min, followed by centrifugation. Afterwards, the samples were frozen at −30°C and the organic phase was transferred to a new tube. The organic phase was evaporated to dryness under nitrogen at 40°C. Acetylation was performed by adding 50 μL of acetic anhydride and 50 μL of pyridine, followed by incubation in the oven at 80°C for 60 min. The acetylation reagents were evaporated under nitrogen at 60°C, and the residue was reconstituted in 100 μL of 75/25 CH_3OH/H_2O and transferred to a vial. To further purify the sample, a single online 3-dimensional LC fraction collection run was performed. More information regarding this LC method can be found in our previously published work.[10] In the context of this study, three fractions were collected, 19NA and the two ERCs, PD and 11oxoEt. All fractions were dried under oxygen-free nitrogen at 60°C. All residues were transferred to a glass vial with 150 μL ethyl acetate, dried under oxygen-free nitrogen at 40°C and reconstituted in an appropriate volume of internal standard solution 5a-ol-Ac (2 μg/mL in 1/1 hexane/toluene) for the GC-C-IRMS analysis. To determine the compounds' $\delta^{13}C$ values, an Agilent 7890A GC equipped with a Gerstel PTV injector was coupled to a Thermo Scientific MAT253 IRMS (Bremen, Germany) with Thermo GC Isolink and a Thermo ConfloIV interface. More information regarding the GC-C-IRMS setup can be found in our previous work.[10]

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3 | RESULTS AND DISCUSSION

A successful IRMS measurement was conducted for 19NA and the ERCs PD and 11oxoEt in each of the 28 samples, despite that some samples had limited urine volume, contained widely varying concentrations of 19NA (range 1.7–4336 ng/mL, adjusted for specific gravity when necessary, i.e., SG > 1.018) and other exogenous steroids, and oftentimes low concentrations of ERCs.

For the blind quality control sample, DoCoLab reported a 19NA $\delta^{13}C = -29.8‰$ (certified 19NA $\delta^{13}C = -29.82‰$).

The collected data is summarised in Tables 1 and 2. Of these 28 samples, only 6 contained 19NA alone (Table 1, highlighted). That is, 22 out of 28 also had an AAF for other exogenous steroids and/or a very high Testosterone/Epitestosterone (T/E) ratio (i.e., T/E ratio >> 4). This indicates that most subjects were users of performance and image-enhancing drugs (PIEDs) and the presence of 19NA

TABLE 1 Summary of data.

Sample number	T/E	Other AAF	19NA[a] (ng/mL)	19NE (ng/mL)	19NA/19NE[b]	A/19NA	19NA ($\delta^{13}C$)[c]	11oxoEt ($\delta^{13}C$)	PD ($\delta^{13}C$)	PD–19NA[d]	11oxoEt–19NA[d]	IRMS[e]	Finding 1[f]	Finding 2[g]
1	42	x	360	74	4.9	18.3	−22.5	−22.0	−22.3	0.2	0.5	NEG	AAF	ATF
2	1.5		1100	300	3.7	1.2	−29.3	−20.0	−19.6	9.7	9.3	AAF	AAF	AAF
3	12		1900	330	5.8	0.4	−22.9	−21.6	−22.4	0.5	1.3	NEG	AAF	ATF
4	13		2500	920	2.7	0.6	−22.9	−21.8	−21.3	1.6	1.1	NEG	AAF	NEG
5	2.2		3.8	1	3.8	496.1	−28.6	−22.7	−22.6	6.0	5.8	AAF	AAF	AAF
6	4.9	x	55	39	1.4	1.1	−25.7	−21.3	−22.1	3.6	4.4	AAF	AAF	AAF
7	81	x	29	4	7.3	75.9	−24.1	−20.6	−21.4	2.6	3.4	ATF	AAF	ATF
8	6.6	x	75	10	7.5	24.0	−22.7	−21.6	−22.2	0.5	1.1	NEG	AAF	ATF
9	58		4	1	4.0	475.0	−27.4	−21.6	−21.5	5.9	5.8	AAF	AAF	AAF
10	113	x	1.7	0.6	2.8	941.2	−27.3	−20.3	−20.5	6.7	6.9	AAF	AAF	AAF
11	3		18	4	4.5	222.2	−24.1	−21.6	−21.3	2.8	2.5	NEG	AAF	ATF
12	0.8		185	80	2.3	9.7	−24.1	−20.6	−20.5	3.6	3.5	AAF	AAF	AAF
13	78	x	144	44	3.3	7.2	−23.3	−20.5	−20.1	3.2	2.8	ATF	AAF	ATF
14	64	x	89	47	1.9	28.1	−23.1	−23.3	−23.9	−0.8	−0.2	NEG	AAF	NEG
15	2.7	x	29	14	2.1	41.4	−25.5	−20.8	−20.7	4.7	4.6	AAF	AAF	AAF
16	0.8	x	141	77	1.8	3.0	−23.6	−23.6	−23.4	0.2	0.1	NEG	AAF	NEG
17	56	x	136	41	3.3	3.0	−20.9	−18.4	−18.0	2.9	2.5	NEG	AAF	ATF
18	1		10.6	6.2	1.7	292.5	−25.3	−22.5	−22.2	3.2	2.8	ATF	ATF	ATF
19	106	x	114	55	2.1	37.7	−25.4	−21.7	−22.7	2.8	3.7	ATF	AAF	ATF
20	32		46	28	1.6	42.4	−22.1	−22.1	−20.7	1.4	0.0	NEG	AAF	NEG
21	48	x	25	9	2.8	304.0	−24.9	−23.6	−22.9	2.0	1.2	NEG	AAF	NEG
22	70	x	28	42	0.7	15.4	−25.0	−23.0	−22.9	2.0	1.9	NEG	AAF	NEG
23	5.1	x	11	2.8	3.9	109.1	−24.6	−21.2	−19.8	4.8	3.4	AAF	AAF	AAF
24	26	x	4336	3204	1.4	2.3	−23.7	−23.1	−22.8	0.9	0.6	NEG	AAF	NEG
25	59	x	650	65	10.0	12.3	−24.1	−23.3	−23.1	1.0	0.8	NEG	AAF	ATF
26	11	x	17	3	5.7	8.5	−23.3	−23.3	−23.1	0.2	0.0	NEG	AAF	ATF
27	4	x	95	45	2.1	16.8	−25.1	−23.4	−22.2	2.9	1.7	NEG	AAF	NEG
28	2.8		6	3.9	1.5	20.8	−27.7	−22.5	−22.6	5.1	5.2	AAF	AAF	AAF
MX017[h]	4		8	1	8.0	212.5	−29.8	−22.8	−22.3	7.4	7.0	AAF	AAF	AAF

Note: Bold/underline, samples containing only 19NA.

Abbreviations: 11oxoEt, 11-oxoetiocholanolone; 19NA, 19-norandrosterone; 19NE, 19-noretiocholanolone; AAF, adverse analytical finding; ATF, atypical finding; ERC-TC, endogenous reference compound–target compound; IRMS, isotope ratio mass spectrometry; PD, pregnanediol.

[a]WADA TD2021NA: red, 19NA > 15 ng/mL; yellow, 19NA < 15 ng/mL.

[b]WADA TD2021NA: red, 19NA/19NE > 3.

[c]'Endogenous/exogenous zone': red - exogenous, 19NA $\delta^{13}C < -26‰$.

[d]WADA TD2021NA: red, $\Delta\delta^{13}C$ ERC–TC > 3.

[e]IRMS finding alone, ignoring 19NA concentration.

[f]Actual overall finding applying TD2021NA using the real 19NA concentration and IRMS results, coloured results required IRMS.

[g]Hypothetical overall finding applying TD2021NA assuming the 19NA was between 2.5 and 15 ng/mL and IRMS results, green – negative, brown – ATF lab opinion.

[h]Quality control sample submitted for IRMS analysis, NMI MX017. Certified 19NA $\delta^{13}C = -29.82‰$.

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TABLE 2 Summary of additional AAFs.

Sample number	Other AAF description
1	Boldenone, drostanolone, oxymetholone, methasterone, tamoxifen
6	Trenbolone
7	Drostanolone, trenbolone
8	Stanozolol, trenbolone, tamoxifen, anastrozole
10	Oxandrolone, dehydrochloromethyltestosterone, trenbolone, ibutamoren
13	Oxymetholone, drostanolone, tamoxifen
14	Boldenone, drostanolone
15	Higenamine
16	Drostanolone, trenbolone
17	Boldenone, letrozole
19	Boldenone, drostanolone, mesterolone, metenolone, methandienone, trenbolone, stanozolol, tamoxifen
21	Boldenone, oxandrolone, methandienone, trenbolone
22	Drostanolone, trenbolone
23	Drostanolone, methandienone, methasterone, trenbolone, tamoxifen
24	Trenbolone, letrozole
25	Boldenone, drostanolone, trenbolone
26	Mesterolone, methandienone
27	Mesterolone, methandienone

Abbreviation: AAF, adverse analytical finding.

in these samples was unlikely, although it cannot be fully excluded, due to factors other than drug administration (e.g., food/supplement contamination, offal consumption, in-situ degradation). The average concentration of 19NA was 432 ng/mL, while the average 19NA/19NE ratio was only 3.4. Six samples had concentrations below 15 ng/mL that would have obliged IRMS analysis if they were athlete anti-doping samples (range 1.7–11 ng/mL, highlighted yellow in Table 1), while the remaining 22 would have been reported directly as an AAF (Finding 1).

Of the 28 samples analysed by IRMS, 23 samples (82%) returned carbon isotope ratio values for 19NA in the endogenous zone ($\delta^{13}C \geq -26‰$). As a result, with respect to IRMS values alone (i.e., based on $\Delta\delta^{13}C$, and assuming hypothetical 19NA concentrations between 2.5 and 15 ng/mL), only nine samples would be reported as an AAF according to WADA TD2021NA (Table 1, Finding 2). A further 11 samples would be categorised as atypical findings (ATFs), including 9 satisfying the criteria in WADA TD2021NA and 2 based on laboratory opinion (as only 1 of 2 ERC–target compound [ERC-TC] combinations fulfilled positivity criteria). The remaining eight samples would be reported negative.

In summary, the overwhelming majority of samples that were analysed were found with endogenous carbon isotope ratios, which is a very significant and consequential finding.

4 | CONCLUSIONS

The collected data indicates that the majority of nandrolone preparations in Australia now have endogenous carbon isotope ratios. This will reduce the capacity of the carbon isotope ratio test to determine whether an athlete has administered nandrolone. The data has highlighted the critical need for laboratories and agencies to adapt their result management processes when a presumptive AAF for nandrolone is determined. These actions may include collecting rapid and well-planned follow-up samples or initiating a preliminary investigation, concurrently with the carbon isotope ratio testing required by WADA.

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