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INTRODUCTION

The World Anti-Doping Agency (WADA) prohibits the use of anabolic-androgenic steroids (AAS) in sport. Despite this prohibition, AAS remains the most prevalent class of compound confirmed in anti-doping samples by WADA-accredited laboratories [1]. Among the most popular and widely detected AAS are nandrolone (N) and testosterone (T), which are prohibited at all times, both in and out of competition [2].

Unfortunately for anti-doping analysis, both of these AAS are pseudo-endogenous as they may also be present naturally. Evidence of N misuse primarily relies on the presence in an athlete's urine of one of its main metabolites, 19-norandrosterone (19-NA), as a consequence of the rapid metabolism of nandrolone [3, 4]. However, WADA stipulates that urinary 19-NA concentration between 2.5 to 15 ng/mL be subjected to further confirmatory analysis [5]. This is because small amounts of 19-NA may be present naturally or formed as degradation products post-sample collection in the urine of males [3, 6] as well as females (especially during pregnancy and ovulation or following norethisterone contraception) [7]. The endogenous production of 19-NA is believed to be a result of the biotransformation of androgens to estrogens by aromatase enzymes [7, 8]. Therefore, detection of small amounts of 19-NA may not be sufficient proof of administration unless the origin of the compound can be determined [5, 7, 8].

The case for T administration is similarly complicated due to the large variation in inter-individual urinary T concentrations (and the concentrations of its metabolites).

Gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) is the established confirmation procedure used by WADA accredited laboratories to distinguish the origin or source of urinary steroids by means of carbon isotope ratio ($^{13}C/^{12}C$) analysis with values expressed in delta (δ) units referenced to the Vienna Pee Dee Belemnite international standard [9, 10].

Synthetic or pharmaceutically-produced steroids are frequently synthesized from soy derived precursors produced by plants, which discriminate significantly against ^{13}C during carbon fixation. As a result, synthetically derived steroids typically have a more negative $\delta^{13}C$ value (range: -27 to -34 ‰) in comparison to normally observed values for endogenous steroids produced by humans (range: -16 to -26 ‰) [11]. The range of ^{13}C content, and hence measured $\delta^{13}C$, for endogenous steroids is a consequence of an individual's diet, which consists of a range of plant and animal sources with varying ^{13}C content [11-13].

In anti-doping analysis, WADA requires the $\delta^{13}C$ value of the target steroid (this may be the administered steroid or a metabolite of the administered steroid) to be compared with that of an endogenous reference compound (ERC) [14]. The ERC (e.g. pregnanediol or 11-keto-etiocholanolone) is a steroid eliminated in urine that is metabolically distinct from the target steroid and therefore not influenced by the administration of the synthetic steroid. As such, its delta value can be considered representative of an individual's diet and metabolism. Following steroid administration, a significant $\delta^{13}C$ difference ($\Delta\delta$) (typically more than 3 ‰) may be observed between ERC and target steroid and can thus be used to confirm administration [15, 16].

However, GC-C-IRMS analysis will be inconclusive if the administered steroid has a similar $\delta^{13}C$ value to that from endogenous steroids. Due to this possibility, many investigations have been conducted on various commercially-available synthetic steroid products (both legitimate and illicit),

to determine if their $\delta^{13}C$ values overlap with those values seen for naturally-produced steroids. Hullstein *et al.* [17] analysed N and T preparations seized in Norway (range -24 to -32 ‰); Forsdahl *et al.* [18] analysed black market T products collected in Austria (range -23 to -29 ‰); and Cawley *et al.* [19] profiled legitimate pharmaceutical and veterinary T products plus illicit T preparations obtained in Australia (range -22 to -32 ‰). To the best of our knowledge, there has been no publication on $\delta^{13}C$ values of steroid products in the UK market. Therefore, we decided to undertake a similar study to investigate the availability of synthetic steroid preparations in the UK that have $\delta^{13}C$ values similar to those of endogenous steroids. Fourteen different N and T preparations obtained from UK pharmacies and custom authorities were analysed to determine identity and carbon isotope ratio.

EXPERIMENTAL

Chemicals and Reagents

All reagents and solvents were of analytical grade. Methanol, ethyl acetate, methyl-*tert*- butyl-ether (MTBE), hydrochloric acid (HCl) and potassium hydroxide (KOH) were purchased from Fisher Scientific (Loughborough, UK). Ammonium iodide (NH_4I) was purchased from VWR International (Lutterworth, UK). Testosterone, Octacosane, dodecane, N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), acetic anhydride, pyridine and ethanethiol were purchased from Sigma Aldrich (Poole, UK). Reference nandrolone was obtained from LGC standards (Teddington UK). Purified water was obtained from ELGA Purelab Maxima (Vivendi Water Systems, High Wycombe, UK).

Steroid preparations

A total of 14 steroid preparations (see Table 1) were analysed. Six were pharmaceutical-grade products purchased from UK pharmacies. The other 8 (seized preparations) were provided by the UK custom authorities through UK Anti-Doping.

Preparation of steroid sample stock solution

Stock solutions of each steroid (250 µg/mL of the ester) were prepared in methanol.

Sample preparation for identification of steroid esters by GC-MS

For identification purposes 10 µL of each steroid stock solution was transferred into a glass tube and evaporated to dryness at 60 ºC for 5 min under nitrogen. Derivatization was carried out by adding 40 µL of the derivatizing solution (MSTFA:NH_4I:ethanethiol, 1000:3:9, v/w/v) to the dried residue, and heating at 60 ºC for 20 min. Once cooled to room temperature, a suitable volume of dodecane was added to yield an (estimated in the case of bulk powders) steroid concentration of 25 µg/mL for GC-MS analysis.

Sample preparation for $\delta^{13}C$ value determination of free steroids by GC-C-IRMS

A method adapted from Forsdahl *et al.* [18] was used. Briefly, 20 µL of steroid stock solution was diluted with 480 µL water. To cleave the esters a hydrolysis step was carried out by adding 1.5 mL of KOH (2 M), and the solution incubated for 2 hours at 60 ºC. The solution was neutralized by adding 1.5 mL of HCl (1 M). Isolation of free steroids was performed using liquid-liquid extraction with MTBE (3 x 2 mL). The combined organic fraction was evaporated to dryness at 60 ºC for 20 min under nitrogen. For the derivatization step, 50 µL acetic anhydride and 50 µL pyridine were added to the dried residue and the solution incubated at 80 ºC for 2 hours, followed by evaporation to dryness at 60 ºC for 20 min under nitrogen. A suitable volume of ethyl acetate was added to yield a signals within the working range of the instrument, with a target signal of 3500 (mV) for GC-C-IRMS analysis (around 40 ng on column).

Gas chromatography mass spectrometry (GC-MS)

Identification of steroid esters was performed using an Agilent GC-MS (Santa Clara, USA) instrument equipped with an automatic sampler, a 7890A gas chromatograph and a 5975C mass selective detector (MSD). Injection port temperature was 280 °C and the sample (1 µL) was injected in splitless mode. Helium was used as the carrier gas with a flow rate of 0.7 mL/min. Chromatographic separation was performed using an Agilent HP-1 capillary column (25 m length x 200 µm internal diameter x 0.11 µm film thickness). The oven temperature programme was 180 °C (1 min), 8 °C/min to 220 °C (0 min), 3 °C/min to 250 °C (0 min) and 14 °C/min to 320 °C (10 min) for a total run time of 31 min. MSD transfer line was set at 280 °C, MS ion source at 230 °C, and MS quadrupole at 150 °C. The MSD was operated in electron ionization mode at 70 eV, with full scan acquisition (mass range m/z 80-800).

Gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS)

The carbon isotope ratio measurements were performed on a Delta V Plus Isotope Ratio MS (ThermoFisher Scientific, Bremen, Germany) coupled to an Agilent 7890A Gas Chromatograph, with a multi-purpose autosampler (GERSTEL GmbH & Co.KG, Germany). Volume of injection is 50 µL using PTV (programmable temperature vaporization) inlet, with a total flow of 154.3 mL/min at 16.4 psi. Chromatographic separation was performed on an Agilent HP-50+ capillary column (50 %-phenyl-methylpolysiloxane, 30 m length x 250 µm i.d., 0.25 µm film thickness). The initial oven temperature was set at 80 ºC for 1.5 min, then increased at 30 ºC/min to 250 ºC, followed by 4.5 ºC/min to 300 ºC and held for 5 min, for a total run time of 23.27 min. Helium was used as carrier gas at a flow rate of 1.3 mL/min. The outlet of the GC column was split between an Agilent MSD 5975C and the IRMS combustion furnace (1:9 ratio), which allowed simultaneous acquisition of full scan mass spectra and IRMS data of eluting analytes. The MSD was operated in full scan acquisition (mass range m/z 40-450). MS source and quadrupole temperatures were 230 ºC and 150 ºC respectively. The IRMS combustion furnace was set to 950 ºC, with copper oxide as the oxygen source. H_2O was removed via a Nafion® membrane. In each analysis run, CO_2 reference gas was pulsed eight times (20 s duration), five at the start and three at the end of the run. The acquisition time for the IRMS was extended beyond that of the GC to 26.7 minutes, which allowed the CO_2 reference gas pulses to be obtained at the end of the run while the GC was cooling down, therefore decreasing the overall runtime. To account for the shift of $\delta^{13}C$ values due to the acetyl moiety introduced during derivatization, a mass balance approach was used [14].

IRMS system suitability checks: stability and linearity

Prior to each batch of samples, a stability test for the instrument was performed by pulsing the CO_2 reference gas 10 times at the same intensity (5,500 mV at m/z 44). To test the IRMS instrument linearity, CO_2 gas was pulsed automatically by the system 7 times over a range of 900-12,000 mV.

Quality control

For every batch, positive (N and T reference standard) and negative (water blank extract) controls

were included. Laboratory-calibrated $^{13}C/^{12}C$ isotopic standard (octacosane, $\delta^{13}C$ = -29.6 ‰) was added in each sample vial as an internal isotopic standard to assess the accuracy of the acquired $\delta^{13}C$ values. The CO_2 reference cylinder itself was calibrated using certified hydrocarbon standards (octacosane and dotriacontane) obtained from Chiron (Trondheim, Norway), and USADA standards (35-1 and 34-2) containing androsterone, etiocholanolone, 5α-androstanediol, 5β-androstanediol and pregnanediol (2 sources), each measured 10 times. The delta value of the CO_2 reference cylinder was set such that the mean difference for all six certified standards between the obtained and certified values was zero. The $\delta^{13}C$ values for all of the steroid preparations were each measured 6 times, and the mean and standard deviation (s.d.) calculated. After every 6 samples injections in each batch, N or T standards were introduced to assure the quality of the analytical measurement. Data acceptance criteria were: retention time of steroid within $\pm$0.1 min of retention time of corresponding standard and a minimum 1,000 mV response at m/z 44 to ensure reliability of $\delta^{13}C$ measurements.

RESULTS AND DISCUSSION

This study consisted of two main steps: The qualitative identification of steroid esters (as TMS derivatives) by GC-MS, followed by the determination of their carbon isotope ratio by GC-C-IRMS (as acetate derivatives).

Steroid identification

All steroids were successfully identified using the applied methodology. As may be expected, all steroids obtained as pharmaceutical preparations contained the expected steroid in ester form. Interestingly, the seized steroids were also all shown to contain the expected steroid, based on the limited information available. For example, several bulk powders simply labelled as NPP were found to contain nandrolone phenylpropionate. No preparation was found to contain significant amounts of any other steroid.

GC-C-IRMS analysis

The measured $\delta^{13}C$ values for the laboratory steroid reference standards (mean ±s.d.) were -30.2 ±0.8 ‰ (n=10) for N and -30.6 ±0.3 ‰ (n=5) for T which were comparable with values of pharmaceutical-grade steroid standards reported in the literature (range: -29.7 to -30.0 ‰) [14, 18].

The octacosane standard analyzed with each sample injection had a mean $\delta^{13}C$ value of -29.3 ±0.6 ‰ (n=84). This is not statistically significant (one sample t-test, $p > 0.05$) compared to the laboratory-assigned value (-29.6 ‰). The range of standard deviation (0.2 - 0.9 ‰, see Table 1) for the replicate injections of unknown steroid samples was within the precision associated with GC-C-IRMS analysis under the specified conditions (<1.0 ‰) [10, 19]. All $\delta^{13}C$ values for steroids reported in this manuscript are for the free steroid, with correction for the influence of the acetate moiety done using a mass balance approach [14].

As GC–C–IRMS analysis involves combustion of a target substance to form CO_2, no spectral information is available to facilitate analyte identification and help identify interfering peaks [10]. Splitting of GC eluents into the MSD and IRMS alleviates this problem by allowing simultaneous acquisition of full scan mass spectra of chromatographic peaks along with isotope ratio analysis [20]. This step is also important to monitor co-elution of interferents with the target steroids which, if present, may alter the measured carbon isotope value. All chromatograms were found to be free of interfering compounds, with an example of the GC-C-IRMS chromatogram presented in Figure 1 and the simultaneously-acquired GC-MS data presented in Figure 2. Additional peaks in the chromatogram where not identified but are likely to result from both the steroid preparation analysed but further work was not performed as it was beyond the scope of the project.

$\delta^{13}C$ values of nandrolone and testosterone preparations

Table 1 shows the $\delta^{13}C$ values of the N and T preparations analysed in this study. All the products acquired from pharmacies displayed $\delta^{13}C$ values (range -27.9 to -32.7 ‰) within the expected range of synthetic steroid preparations (less than -27 ‰) and can therefore be considered consistent with

production from soy or a similar source. This range was also similar to those reported in other studies investigating legitimate pharmaceutical preparations, i.e. -27.0 to -28.4 ‰ [21], -27.6 to -30.6 ‰ [22] and -27.4 to -31.8 ‰ [19].

As highlighted in Table 1, four N preparations showed $\delta^{13}C$ values in the endogenous range, all corresponding to preparations that were seized by UK custom authorities. In contrast, a similar investigation by Hullstein *et al.* [17] on N preparations (n = 22) seized in Norway demonstrated that almost all $\delta^{13}C$ values appeared in the exogenous range (< -27.0 ‰), with only one preparation identified to be close to the endogenous range (-26.7 ‰). These "endogenous-like" preparations are unlikely to result from soy plant derived precursors, or indeed any other plant material which uses the same photosynthetic pathway (C3). An alternative source material for these steroids may be that they are derived from sisal. Sisal (*Agave sisalana*) is a widely grown cash crop originating in Mexico but now prevalent all over the world. Steroids derived from plants use sapogenins as the starting material and hecogenin, a sapogenin available in large quantities from the waste of sisal plants is known to be used for synthesis of cortisol [23, 24]. This is particularly important as sisal unlike most plants does not use the C3 (or indeed C4) photosynthetic pathway but instead uses the crassulacean acid metabolism (CAM) pathway. This photosynthetic mechanism results in less discrimination of ^{13}C during carbon fixation and therefore will produce delta values that are less negative than soy-derived steroids but more like those observed for the four "endogenous-like" N preparations seen in this study. Though little research has been done on the carbon isotope ratio signature of sisal plants, research on the carbon delta value of other CAM plants such as pineapple has been undertaken and offers some support to the hypothesis proposed here [25].

None of the T preparations in this study had $\delta^{13}C$ values within the endogenous range. Though this is in contrast to the published data, it has to be considered that this may be a consequence of the small sample size in this study (n = 5) and the fact that four of the five preparations were sourced from pharmacies [17-19, 22]

In doping-control analysis, detecting the misuse of steroids produced naturally by humans (i.e. T and 19-NA) is regarded as one of the most challenging tasks due to the difficulty in proving their administration [12]. The availability of synthetic T and N preparations with endogenous $\delta^{13}C$ values on the market as has been shown in this study and those of others [17-19, 22] further complicates substantiating evidence of their misuse. This is because, if these steroid preparations are administered, analysis by GC-C-IRMS will yield urinary steroids exhibiting $\delta^{13}C$ values that cannot be differentiated from endogenous origin. Based on WADA guidelines, this may be reported as an inconclusive or atypical finding rather than an adverse analytical finding [5, 14, 26]. Other factors such as steroid dosage and timing of urine collection post-administration [22] could also influence a negative or positive outcome since the $\delta^{13}C$ value will normalize to the athlete's baseline level after a washout time, that is either a normalisation of the $\delta^{13}C$ value back to endogenous in the case of T [27] or a reduction in 19-NA concentration such that it is below the measurement capability of the assay.

On the other hand, misuse of steroid preparations that have $\delta^{13}C$ values further into the exogenous range (less than -29 ‰) remain easy to detect. For instance, administration of the most ^{13}C-depleted

products (-32.7 ‰) represent the most likely to yield positive results by GC-C-IRMS analysis as demonstrated in previous studies [28].

CONCLUSION

In conclusion, analysis of $\delta^{13}C$ values for fourteen preparations containing N and T revealed that four N preparations, all seized material, displayed a $\delta^{13}C$ value that overlaps with the values measured for endogenous steroids (range -26 to 16 ‰). Administration of these preparations would be challenging to prove from a doping-control context because the corresponding urinary metabolites would exhibit $\delta^{13}C$ values within the range of endogenous steroids, hence yielding a negative or inconclusive result by GC-C-IRMS analysis. It may be that the analysis of additional isotope signatures, such as hydrogen may offer improved discrimination but this will require further research. In such cases, the timing of sample collection would need to be close to administration of the N preparation to detect the parent compound rather than rely on such confirmatory analysis.

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Figure 1: GC-C-IRMS chromatogram of nandrolone preparation #9 (b), in its acetylated form. The top trace shows the ratio of m/z 45/44. The bottom trace (m/z 44) shows the chromatogram peaks of nandrolone (1002 s) and octacosane within analysis standard (721 s).

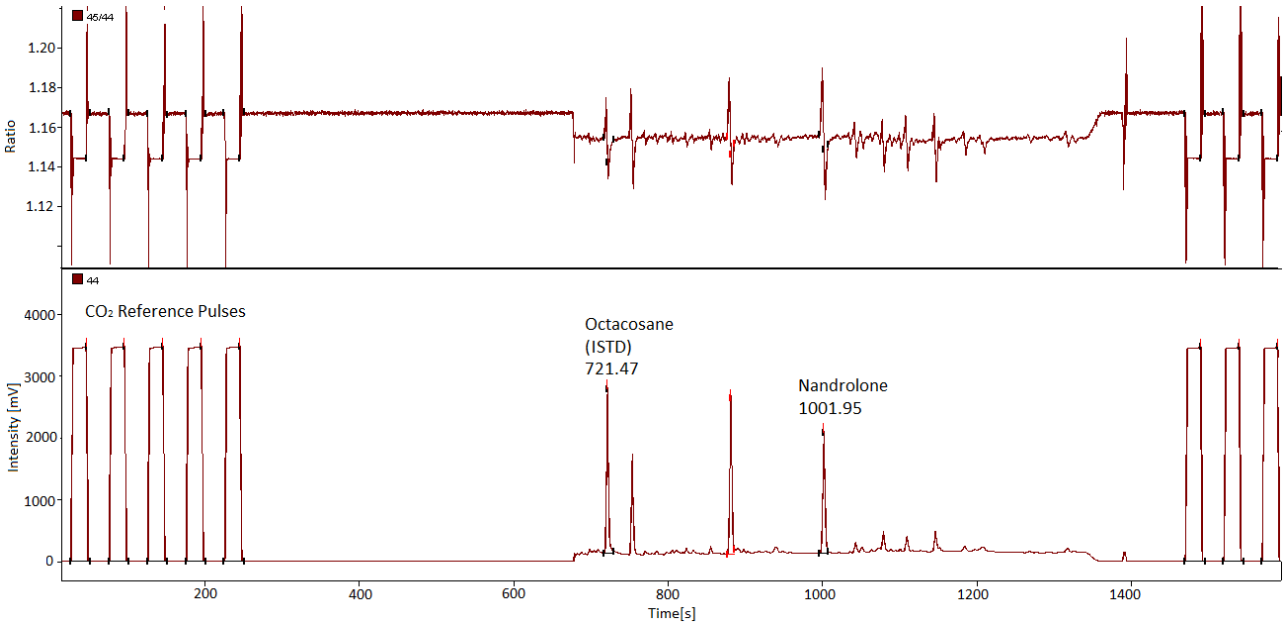

Figure 2: The corresponding GC-MS chromatogram & mass spectrum of nandrolone preparation #9 (b), in its acetylated form.