Review

Drug Testing and Analysis

Received: 19 February 2012 Revised: 6 May 2012 Accepted: 8 May 2012 Published online in Wiley Online Library: 13 July 2012

(www.drugtestinganalysis.com) DOI 10.1002/dta.1378

Complementary stable carbon isotope ratio and amount of substance measurements in sports anti-doping

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The detection of steroids originating from synthetic precursors against a background of their chemically identical natural analogues has proven to be a significant challenge for doping control laboratories accredited by the World Anti-Doping Agency (WADA). The complementary application of gas chromatography–mass spectrometry (GC-MS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) has been demonstrated to provide specific detection of endogenous steroid misuse for improved anti-doping analysis. Markers of synthetically derived steroids are reviewed on the basis of abnormal urinary excretions and low ^{13}C content. A combinatorial approach is presented for the interpretation of GC-MS and GC-C-IRMS data in the anti-doping context. This methodology can allow all relevant information concerning an individual's metabolism to be assessed in order to make an informed decision with respect to a doping violation. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: endogenous steroids; doping control; gas chromatography–mass spectrometry (GC-MS); steroid profile; gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)

Introduction

The problem of doping in sport represents a public health and equity issue. The detection of doping practices requires the development of reliable, non-invasive, cost-effective analytical methods. Endogenous steroid abuse presents a particular problem for doping control laboratories to determine the origin of steroids as being from the *body* or the *bottle*. In an effort to increase circulating levels of biologically active steroids, the abuse of prohormones – steroids capable of being metabolized to testosterone (T; androst-4-ene-17β-ol-3-one) and dihydrotestosterone (DHT; 5α-androstane-17β-ol-3-one) – has provided a significant challenge to identify what specific steroid has been administered. In turn, this has proved to be a stimulus for analytical research.

The study of endogenous steroid metabolism rapidly evolved after 1934, when Butenandt and Tscherning revealed dehydroepiandrosterone (DHEA; androst-5-ene-3β-ol-17-one) to be excreted in human urine in conjugated form.[1] It was a further 10 years, however, before Munson *et al.*[2] succeeded in isolating and characterizing the sulfoconjugate (DHEA-S). Despite the fact that DHEA-S is one of the principal excreted metabolic products of the adrenal cortex, the biological significance of this conjugate remains unknown. The possibility for DHEA to increase performance prescribes its inclusion in the list of prohibited substances published by the World Anti-Doping Agency (WADA).[3] In addition, health concerns surrounding hepatic toxicity associated with DHEA administration further emphasizes the need for prohibition.[4]

Androstenedione (ADIONE; androst-4-ene-3,17-dione), like DHEA, is an endogenous steroid said to be a prohormone due to the possibility that it may be converted to testosterone in the body.[5] The chemical relationship of ADIONE to T was apparent from the initial report of its synthesis by Ruzicka and Wettstein in 1935.[6] By the end of the 1990s it had been revealed that East German athletes were treated with intranasal pulses of ADIONE in an attempt to increase T levels and thus athletic performance.[7,8] Since it was banned by the International Olympic Committee in 1997, ADIONE has attracted significant public attention, most notably in 1998 when US major league baseball player Mark McGwire admitted to using ADIONE while hitting the world record number of home runs. In a wide-ranging, yet concise, commentary on ADIONE abuse Yesalis discusses some of the medical, legal and societal implications.[9]

4-androstenediol (4-ADIOL; androst-4-ene-3β,17β-diol) and 5-androstenediol (5-ADIOL; androst-5-ene-3β,17β-diol) are banned by WADA on the premise that they may be converted to biologically active T.[3,10] The structural similarity between 4- and 5-ADIOL provides an opportunity to investigate isotopic differences in the common metabolites formed from both precursors.

The detection of administered synthetic steroid analogues has created new areas of research in recent years. Before 1982, only the use of exogenous substances (i.e. those foreign to the body) had been banned, with a positive result requiring unequivocal proof provided by mass spectrometry that the banned substance and/or its metabolites were present in a urine sample.[11] The mere presence of steroids known to be endogenous in the urine of an athlete obviously cannot constitute proof of administration. Investigations of gas chromatography–mass spectrometry (GC-MS) steroid profiles following endogenous steroid administrations

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have been performed relative to normal intervals from reference populations to raise suspicion of abuse.[12–17] Early research was specific to detect administration of T since it is thought to be the principal androgen capable of conversion to the more active DHT. In 1983, Donike *et al.*[18] proposed to detect T administration by monitoring the urinary testosterone glucuronide to epitestosterone glucuronide ratio (T/E) – epitestosterone (E; androst-4-ene-17α-ol-3-one) being the inactive epimeric by-product of T biosynthesis. Administration of T reduces its natural production in the body due to a negative feedback mechanism involving the suppression of luteinizing hormone that activates the natural production of T. The natural production of E is also reduced resulting in an increase in the urinary T/E. T and E have been shown to exist in approximately equal amounts in humans such that a normal T/E is expected to be 1.[13,18,19] This has led WADA to define a T/E of greater than 4 as the dividing line between the upper limit of normal T/E values and those resulting from administration of T – thereby accounting for natural ratio variations.[20] A small proportion of the population exists, however, with a natural T/E greater than 4, most likely due to low excretion of E glucuronide relative to its sulfoconjugate.[21] These exemptions to the threshold of guilt that do not result from T abuse have contributed to considerable uncertainty when confirming doping.[22–24] A natural cause of an elevated T/E may be highlighted by longitudinal testing of an athlete over a period of months. WADA's technical document for endogenous steroid analysis defines natural variation of T/E in males to be no more than 30%.[20] A larger value (60%) is allowed for females due to the low urinary excretion of T and E expected from this population that are close to the analytical limits of detection, and variation during the menstrual cycle.[20] Any greater change in the T/E of the athlete over time would confirm the administration of T since the T/E for individuals has been shown to be stable.[13,18,19] Regardless of this, the interpretation of altered T/E remains complex.

Doping control laboratories accredited by WADA have been confronted with the challenge to develop methods and establish criteria that allow endogenous steroids to be distinguished from their synthetic analogues in the urine of athletes without the need for longitudinal studies involving suspicious athletes. While administered synthetic steroids are chemically identical to those produced and excreted by the body, there are small differences in the ^{13}C to ^{12}C ratio of synthetic analogues that can be measured using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS).[25–27] The expression of stable carbon isotope ratios for steroid analysis in terms of $\delta^{13}C$ (‰) values has been summarized in recent papers.[28–30] Differences in steroid ^{13}C content reflect isotopic fractionation patterns that are, in the first instance, associated with photosynthesis.[31,32] The ^{13}C depleted content of ingested substances[33,34] is carried forward with metabolism to the excretion of urinary steroids.[34–39]

During the development and validation of stable carbon isotope ratio analysis for doping control, a T/E value greater than 4 measured in the GC-MS steroid profile would initiate investigation by GC-C-IRMS. However the GC-C-IRMS results were then considered to be somewhat discrete from the original profile. Subsequent investigations reviewed herein have promoted the value of a complementary GC-MS/GC-C-IRMS strategy. It is prudent to first consider the capabilities of GC-MS and GC-C-IRMS profiling methods for doping control before such a combinatorial approach can be accepted.

GC-MS steroid profiling

Advanced GC-MS steroid profiling strategies can screen all urine samples entering the laboratory for the presence of markers that are attributed to specific steroid administrations, while eliminating those of a non-suspicious nature. This may require as little as a 3 ml sample volume. Isolation of urinary steroids from their glucuronide moieties and GC-MS parameters for their analysis as trimethyl-silyl (TMS) enol-ethers has been adapted by anti-doping laboratories from previously reported methods.[12,18,24,40,41] While the analysis of steroid glucuronides is considered routine using β-glucuronidase from *E.coli*, the development of a robust and reproducible analytical method for urinary analysis of steroids originating from their sulfate conjugates has not proved to be simple. Chemical hydrolysis is preferable to enzymatic methods as it results in quantitative cleavage of the sulfate moiety. Many of the published chemical hydrolysis methods[42–44] relating to steroid sulfates are adaptations of the method described by Burstein and Lieberman in 1958.[45] For the purpose intended, however, these have been problematic to implement due to matrix effects.[46] The solution lay in purification of steroid sulfoconjugates prior to hydrolysis.[47,48]

A further challenge for endogenous steroid profiling is the response of the mass selective detector to compounds displaying considerably different excretion patterns.[49] This is particularly true for 'high-load' steroids that appear as fronting peaks with distorted ion abundances due to saturation effects. Accuracy in the quantification of urinary steroids ranging in concentration from 10 ng/ml to 10000 ng/ml can be achieved with desired linearity by analyzing samples with and without suitable dilution factors applied. Meaningful metabolic interpretations should be supported by quality control parameters that demonstrate fitness-for-purpose of the applied analytical method with respect to extraction efficiency, hydrolysis of steroid glucuronides and sulfoconjugates, and derivatization.

The potential of chemometric evaluation of the GC-MS steroid profile was reported in 1995 by the Norwegian anti-doping laboratory.[49] This approach used multivariate partial least squares regression of peak normalized data to distinguish samples positive for doping from the negative population. The key to such strategies lies in establishing reference ranges for steroid metabolites that are representative of the athlete population. This has been addressed for the Caucasian population with the work of Van Renterghem *et al.*[50] who profiled the concentrations of 29 endogenous steroids and 11 ratios from 2027 male and 1004 female athletes.

GC-C-IRMS methodology

The ability to measure isotope distribution at natural abundance with great accuracy and high precision has increased the application of GC-C-IRMS in recent years.[51] Implementation of GC-C-IRMS analysis into the XVII Nagano Olympic Winter Games testing programme represented a milestone for the technique in doping control. At the event, 450 samples providing abnormal GC-MS steroid profiles were checked by GC-C-IRMS to confirm there was no endogenous steroid abuse. This exercise provided the first in-competition elite athlete $\delta^{13}C$ reference intervals for 5α-androstane-3α,17β-diol (ααβ-diol) and 5β-androstane-3α,17β-diol (βαβ-diol) corrected for the measurement of diacetate derivatives.[34] In contrast to early studies investigating GC-C-IRMS

analysis of steroid metabolites from the glucuronide fraction following liquid-liquid extraction, the Japanese anti-doping laboratory employed solid-phase extraction (SPE) techniques with pH gradient elution. This allowed the determination of $\delta^{13}C$ values of steroids such as DHEA obtained from the sulfoconjugate fraction. Conversion of steroid metabolites to their acetate derivatives was another important issue confronted by this group. Acetylation, while an effective means of improving chromatographic resolution of steroids by increasing their volatility, may result in fractionation. For each acetate group, two carbon atoms have been added to the steroid molecule, thereby altering its native $\delta^{13}C$ value. Additionally, kinetic isotope effects predominate when the reaction does not achieve completion.[52] To compensate for these problems an internal standard was added pre-derivatization to correct steroid $\delta^{13}C$ values for the presence of acetate moieties.[34]

Valid $\delta^{13}C$ measurements require isotopic fractionation during sample preparation to be excluded.[53] Flenker *et al*.[54] reported $\delta^{13}C$ values of endogenous steroid metabolites obtained without derivatization. Semi-preparative high performance ion trap mass spectrometry (HPLC) steroid purification methods combined with optimum GC-C-IRMS conditions produced excellent chromatographic resolution required for accurate reporting of $\delta^{13}C$ values. While developing the use of HPLC purification, this group investigated the fractionation of steroids this method may provide as a potential source of error. While careful peak definition in GC-C-IRMS analysis is a pre-requisite for accurate results, chromatographic separation by HPLC was demonstrated to significantly alter ^{13}C content of a steroid when the entire peak was not collected. Full scan GC-MS analysis of pre- and post-analyte HPLC collections, with comparison to authentic reference materials is considered to be the best way to determine that a complete steroid peak is analyzed by GC-C-IRMS.[28]

Another major source of $\delta^{13}C$ variation is diet. The basis of the GC-C-IRMS technique to distinguish endogenous steroid metabolites from their synthetic analogues is, by nature, derived from the diet of an athlete. Diets can vary greatly with culture and sport, and this may be accounted for in GC-C-IRMS analysis by measuring the $\delta^{13}C$ value of a 'background marker' steroid that is not involved in the androgen metabolic pathway. Such steroids are referred to as endogenous reference compounds (or ERCs). The use of ERCs was addressed in studies by Becchi[35] and Shackleton[36,37] that determined the $\delta^{13}C$ value of pregnanediol (PD; 5β-pregnane-3α,20α-diol) to account for the diet of individual athletes. Aguilera *et al*.[38] proposed cholesterol as an ERC; however, its urinary origins are not well understood. Large deviations in $\delta^{13}C$ values between androgen metabolites and an ERC are proposed to substantiate a doping violation, together with the absolute $\delta^{13}C$ values of the androgen metabolites themselves. The reporting of such deviations has also been a source of intense discussion in the doping control community. Early studies used reference intervals to provide the $\delta^{13}C$ mean of ααβ-diol and βαβ-diol as a ratio to $\delta^{13}C$ PD, with a limit value greater than 1.5‰ representing a doping violation. Flenker and Schänzer demonstrated that expressing $\delta^{13}C$ values as a ratio provides a flawed interpretation of $\delta^{13}C$ deviations.[55] Instead, the difference of an ERC $\delta^{13}C$ value from that of selected androgen metabolites represents the most effective reporting method. These $\Delta\delta^{13}C$ values provide a mathematically sound basis to identify doping cases.

Of course, in the ongoing anti-doping battle between the scientists and the cheats, the weaknesses of any analysis method need to be considered. Just as the use of T/E values raised the possibility of offenders co-administering E with T, GC-C-IRMS analysis methods based on $\Delta\delta^{13}C$ values were thought to be limited by co-administration of ERC's. Saudan *et al*.[56] demonstrated ^{13}C depletion of PD following oral administration of its precursor, pregnenolone. This investigation led to 5α-androst-16-ene-3α-ol being proposed as a suitable ERC, when precursors to PD have been administered.[57] Further work was conducted by Piper *et al*.[58] to investigate the effect of pregnenolone administration on additional target analytes, originating from both the glucuronide and sulfate fraction. Of relevance to this review, the group used combined GC-MS and GC-C-IRMS profiling of a defined metabolite set to show that only PD and 5β-pregnane-3α-ol-20-one displayed significant increases in urinary excretion together with ^{13}C-depletion of both conjugated forms.

In 2003, a United States Anti-doping Agency (USADA) research symposium recommended development of steroid isotopic internal standards to harmonize reported values.[59] In response the group at Cornell University led by Brenna has provided isotopically calibrated steroids to anti-doping laboratories that can be analyzed under GC conditions identical to sample steroids.[60] The availability of these reference materials together with fit-for-purpose validation strategies such as the linear mixing models implemented by Piper *et al*.[29] provide confidence in the accuracy and precision of $\delta^{13}C$ values within and between anti-doping laboratories.

$\delta^{13}C$ reference intervals

A number of profiling studies have been conducted to investigate the variation in, and establish reference intervals for, $\delta^{13}C$ values of urinary excreted endogenous steroid metabolites and discuss the consequences of these for doping control.[29,34,61–65] Results from more than 1500 individuals have proposed a $\delta^{13}C$ reference interval from −16.7‰ to −25.8‰ comprising two groups derived from C-3 and C-4 diets, while $\delta^{13}C$ values less (i.e. more negative) than −26.8‰ are assumed to have originated from synthetically ^{13}C depleted steroid precursors. Importantly, each study has substantiated the need for the reporting of $\Delta\delta^{13}C$ values to normalize $\delta^{13}C$ measurements of target steroids for inter-individual variations and thereby confirm synthetic steroid misuse. By definition, a $\Delta\delta^{13}C$ value is determined from the choice of target steroid and ERC, both of which have been shown to influence decision making in relation to criteria prosecuting doping violations.[29,63]

Stable carbon isotope ratio methodology used in doping control presumes that physiological variations of such enzymes do not significantly alter $\Delta\delta^{13}C$ values. Lüdke *et al*.[66] investigated this assumption to demonstrate the existence and magnitude of stable carbon isotope discrimination by human 3-hydroxy-3-methylglutaryl-CoA reductase to be within 0.5‰ when comparing C_{19}- and C_{21}-steroid hormones, which is itself within the measurement uncertainty estimated for GC-C-IRMS results.[67] Nonetheless, it will be reviewed herein that different steroids, although originating from the same metabolic pathway (Figure 1) do not necessarily exhibit identical stable isotope signatures.

Dehydroepiandrosterone (DHEA)

Oral administrations of DHEA to male volunteers have provided the opportunity to observe changes in urinary steroid excretion and interpret pharmacokinetic behaviour.[14,16,37,68–70] The magnitude

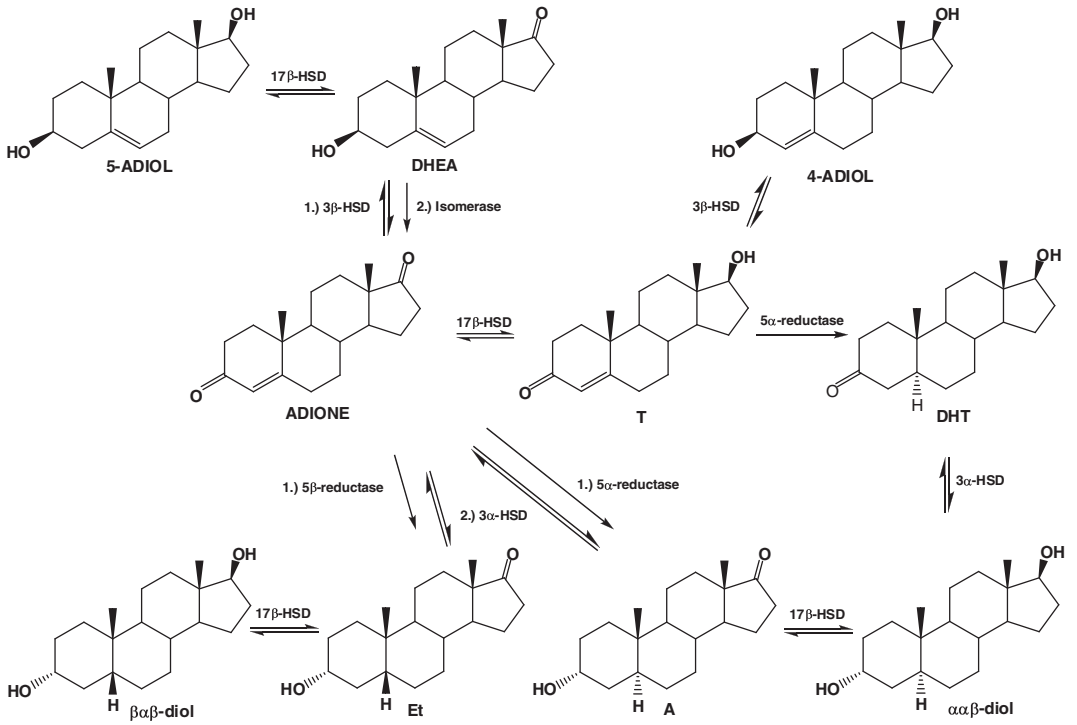

Figure 1. Endogenous steroid biosynthetic pathway (HSD = hydroxysteroid dehydrogenase). Adapted from Van Renterghem *et al.*[82]

of such changes, however, is highly dependent on homeostatic (i.e. baseline) levels. When monitoring metabolite ratios, the direction of alterations will also be influenced by the normal metabolic flux as determined by enzymatic reactions. The best example of such phenomenon is observed for T/E values that are elevated following DHEA administration for individuals with baseline values greater than 1,[68,69] but constant or even reduced for those with initial T/E less than 1.[14,16,71] In a foundation article for the application of stable carbon isotope ratio analysis in doping control, Shackleton *et al.*[37] reported the complementary use of GC-MS and GC-C-IRMS measurements of 5-ADIOL to confirm DHEA administration, distinct from T, E, or DHT misuse.

Pharmacological differences in the metabolism of administered DHEA are observed with the distinct times of maximum excretion for androsterone (A; 5α-androstane-3α-ol-17-one) glucuronide (A-G) and etiocholanolone (Et; 5β-androstane-3α-ol-17-one) glucuronide (Et-G). Consistent for all studies of DHEA administration has been a significantly perturbed A-G to Et-G ratio. Following single oral doses an initial peak in A-G was followed by a rapid return to baseline, while Et-G remained elevated for up to 72 h.[16,71] Similarly, the behaviour of ααβ-diol and βαβ-diol measured in the glucuronide fraction reflects the change in concentration of A-G and Et-G. The preference for prolonged production of 5β-reduced metabolites is most likely due to pharmacokinetic mechanisms involving the location of 5β-reductase in the cytoplasm of hepatocytes, in contrast to 5α-reductase located in the endoplasmic reticulum.[72] A predominance of phase I metabolic activity can be explained by dual-compartment pharmacokinetics for excess DHEA to be absorbed by peripheral tissues prior to delayed release in conjunction with the suppressed steroidogenic state resulting from negative-feedback. Separately or in addition, the double surge effect can be explained by phase II mechanisms where hydrophilic steroid conjugates are concentrated in bile and delivered to the gastrointestinal tract for hydrolysis to release the free steroid. This can be reabsorbed and the cycle repeated in an enterohepatic process.[73] Predominant conjugation of DHEA to form its corresponding sulfoconjugate and subsequent hydrolysis of the highly labile sulfo-moiety is likely to represent a favourable candidate for enterohepatic circulation.[74–76] Sulfoconjugation results in increased androsterone 3α-sulfoconjugate (A-S) and etiocholanolone 3α-sulfoconjugate (Et-S) excretions in tandem with their respective glucuronides. A study conducted by the authors showed the percentage of A-S derived from total A (G + S) excreted remained constant while the proportion of excreted Et-S increased, thereby demonstrating a metabolic preference for the sulfoconjugation of 5β-reduced steroid metabolites compared to their 5α-reduced analogues.[71] These excretion amounts were accompanied by ^{13}C-depletion patterns with differences between δ^{13}C Et and δ^{13}C A increasing by more than 3‰ following the administration of DHEA.[77] Piper *et al.*[30,48] further investigated this phenomenon by measuring δ^{13}C values of urinary androstanediols. Different pool sizes of ααβ-diol and A, together with greater access to 5α-reductase in different tissues enables rapid metabolism of administered DHEA via this route with ααβ-diol as intermediate. In contrast, δ^{13}C values of βαβ-diol are only mildly influenced raising doubts that it is the only intermediate to production of Et.

Theoretically, the most effective detection strategy for DHEA abuse would take advantage of high urinary DHEA-S excretion to provide δ^{13}C evidence of illegal administration. Interestingly, however, two independent studies have shown the δ^{13}C value of DHEA-S not to reach the δ^{13}C value of the administered DHEA,[48,71] which can be explained by the large endogenous DHEA-S pool.[78,79] Generally, steroid sulfoconjugates provide δ^{13}C values that are higher (i.e. ^{13}C-enriched) than their respective glucuronides. The unexpectedly high δ^{13}C values of DHEA-S provide little advantage for doping control over the

use of δ^{13}C Et-S or indeed δ^{13}C Et-G. Regarding the latter, both cited studies reported lower δ^{13}C values of Et-G relative to the administered DHEA, thereby demonstrating *in vivo* ^{13}C fractionation.[48,71] Concerning sulfoconjugate analysis, Piper *et al*.[48] concluded that only epiandrosterone (5α-androstane-3β-ol-17-one) could potentially prolong the detection time for DHEA misuse. As such methods are time-consuming, they cannot be implemented in routine analysis but could be used as a confirmation procedure in cases where δ^{13}C values of glucuronide-derived steroids provide inconclusive results.

In addition to the measurement of known target analytes, complementary analysis platforms can be used to search for novel compounds. Changes in the abundance of metabolites along with the measurement of ^{13}C content may provide evidence of diagnostic products arising from specific endogenous steroid administrations. Work conducted by the authors reported the unexpected presence, following DHEA administration, of a previously unidentified steroid.[80] Interest surrounding the GC-C-IRMS peak eluting slightly before the terminal androgen metabolites – A-G and Et-G – was increased with the observed change in abundance according to the dosing regime of multiple DHEA administrations. By way of its presence in GC-C-IRMS traces obtained with limited molecular sensitivity the excretion amount was itself a clue to the potential of 3α,5-cyclo-5α-androstane-6β-ol-17-one (3α,5-cyclo) for doping control. Cyclosteroid formation from steroid sulfoconjugates was demonstrated by the urinary excretion of 3α,5-cyclo correlated with that of DHEA-S.[71] The screening of athlete's urine samples for DHEA abuse using a limit for 3α,5-cyclo of 100 ng/ml – corrected to specific gravity of 1.020 was proposed. There is compelling evidence supporting the use of markers, both metabolic and non-metabolic (formed after urine has passed into the bladder), in screening and confirmation of androgen abuse. In this case the excretion of 3α,5-cyclo reflects the concentration of DHEA-S and circumvents the need for removal of the sulfoconjugate moiety. This approach has been adopted by anti-doping laboratories [81,82] but its value has also been questioned when present in degraded urine samples.[83]

The conventional approach used by anti-doping laboratories to distinguish endogenous steroids from their synthetic analogues has relied on the support of population-based reference intervals. Further to the potential of combinatorial methodologies, is the alternative of subject-based reference intervals for androgen metabolites and their corresponding ratios using Bayesian inference techniques as the number of records for an individual athlete increase.[84,85] This led to screening limits for 7βOH-DHEA (126 ng/ml) and 16αOH-DHEA (37 ng/ml), together with their respective ratios to E, being proposed as additional biomarkers of DHEA administration that are reported to increase the sensitivity of longitudinal testing.[70,86] It is expected that the δ^{13}C values of these markers will provide confirmatory evidence of DHEA misuse in future.

The investigation of biomarkers for DHEA abuse prompted the methodology to 'look outside the metabolic box' that doping control laboratories may find themselves constrained to in their research (Figure 2). This is not to be dismissive of the literature, rather it serves to emphasize the value of early literature – such as the review performed by Bradlow[87] – to research involving endogenous steroids.

Androstenedione (ADIONE)

Similarly to DHEA, studies have shown the administration of ADIONE to increase urinary T/E values in subjects with normal (≈1) or naturally high (>1) initial values but not to increase T/E

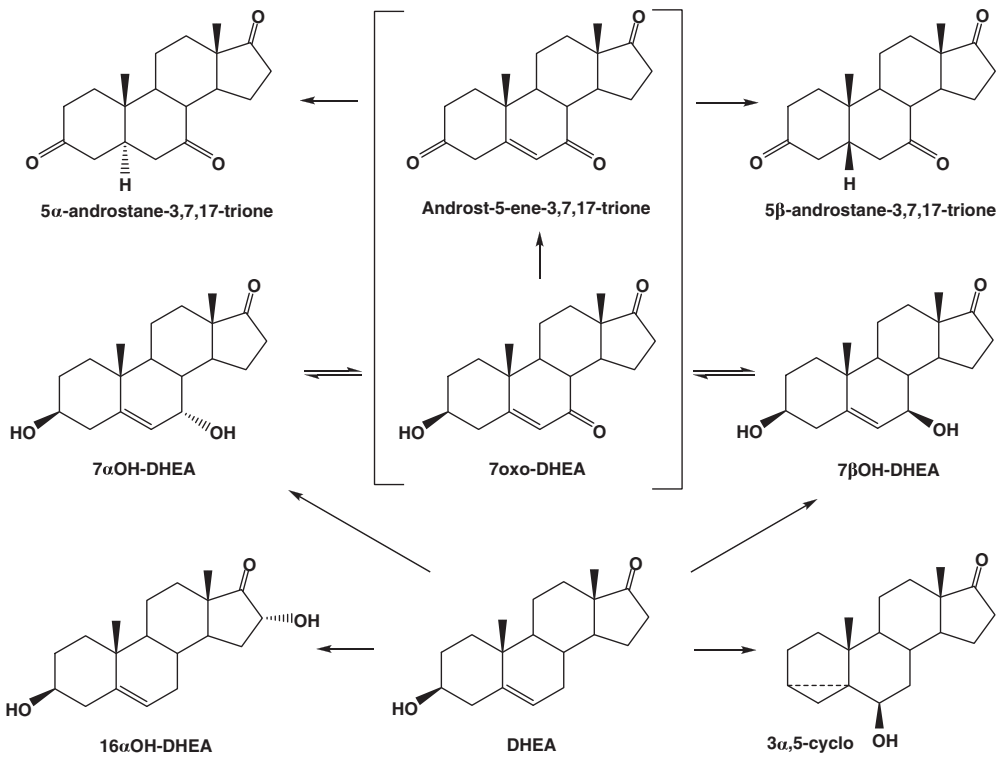

Figure 2. Biomarkers of DHEA and 7oxo-DHEA misuse.[24,70,80–82,86,118]

values in subjects with naturally low (<1) initial values. An overload of A-G and Et-G was proposed as the most effective screening marker of ADIONE administration.[15,17,71] Using any of these indicators, the challenge for doping control laboratories lies in specific identification of ADIONE abuse, thereby distinguishing it from other prohormones such as DHEA (Figure 3). For this purpose, Lévesque and Ayotte[88] made an important contribution with the characterization of several C_6-hydroxylated metabolites – 6αOH-ADIONE, 6βOH-A, 6βOH-Et and 6βOH-epiA – that originate from ADIONE administration in both the glucuronide and sulfoconjugated steroid fractions. Although they can be effective in identifying ADIONE administration in the routine GC-MS steroid profile, the relatively low urinary excretion of these C_6-hydroxylated metabolites limits the use of GC-C-IRMS to specifically confirm ADIONE administration based on depleted ^{13}C content. Extending the biomarker approach reported by the Canadian anti-doping laboratory,[88] a predictive mass spectrometry investigation was used to identify 4OH-ADIONE (androst-4-en-4-ol-3,17-dione; also called formestane) that is excreted in larger amounts, thereby enabling complementary GC-MS/GC-C-IRMS analyses.[89] This followed the work of van de Kerkhof who showed C_4-hydroxylation to be a metabolic pathway induced by ADIONE administration.[90] A screening limit for 4OH-ADIONE of 40 ng/ml has been proposed by independent studies based on reference intervals that demonstrated its endogenous nature.[89–91] In 2010, WADA established a threshold for urinary 4OH-ADIONE excreted as glucuronide of 100 ng/ml, above which stable carbon isotope ratio analysis would be used to establish its origin.[92]

4-Androstenediol (4-ADIOL)

Excretion patterns for monitored steroid metabolites following oral administration of 4-ADIOL are reported to be similar to ADIONE with rapid elimination of the administered substrate.[15,71] This occurred via first-pass metabolism that was demonstrated by an increased excretion of monitored steroids and no presence of urinary 4-ADIOL. Further to the use of the T/E measurement, steroid excretions expressed as relative ratios can account for diurnal excretion variations originating from the circadian rhythm, while providing information concerning natural and altered selection of metabolic pathways. Limitations are found, however, for the universal application of steroid ratios to identify specific endogenous steroid abuse. A/T values that may be used as an additional indicator of T administration,[18,93] can be subject dependent. While subjects with a typical steroid profile may display suppressed A/T values with 4-ADIOL administration, A/T values obtained from other subjects can show significant elevation.[71]

Sulfoconjugate analysis has provided $\delta^{13}C$ values of Et-S and A-S that were approximately 5‰ lower than their respective glucuronide analogues following a single oral 100 mg 4-ADIOL administration.[71] This distinctive ^{13}C-fractionation pattern of Et-S has the potential to increase the post-administration detection period of 4-ADIOL administration. These findings may be explained by sulfoconjugation increasing water solubility and protein binding, thereby influencing transportability. Due to the high binding affinity of sulfoconjugated steroids with serum proteins such as albumin, they generally have slower clearance rates from the blood compartment than their glucuronide analogues and therefore can provide longer periods of detection post-administration of 4-ADIOL abuse.

Continuing the development of strategies for complementary analysis of novel steroid biomarkers by GC-MS and GC-C-IRMS, recent research demonstrated the potential of androst-2,4-diene-17-one and androst-3,5-diene-17-one to specifically identify 4-ADIOL administration (Figure 4).[94] Further to the 3α,5-cyclo marker of DHEA administration, the measurement of urinary by-products originating from ingested synthetic material expands

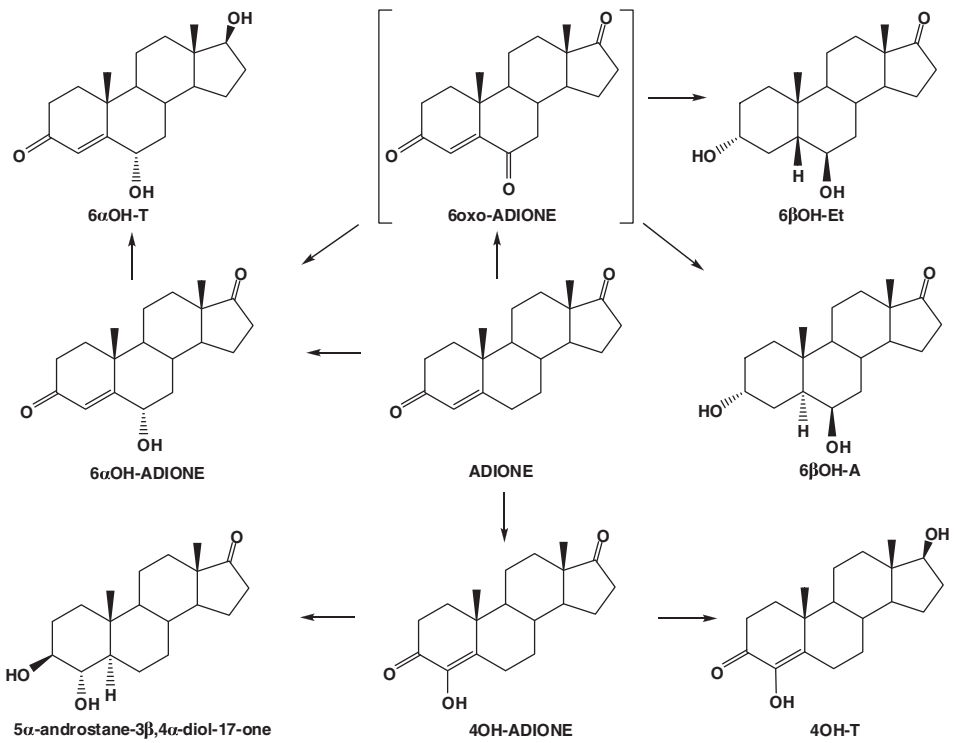

Figure 3. Biomarkers of ADIONE, 4OH-ADIONE and 6oxo-ADIONE misuse.[82,88–91,121–123]

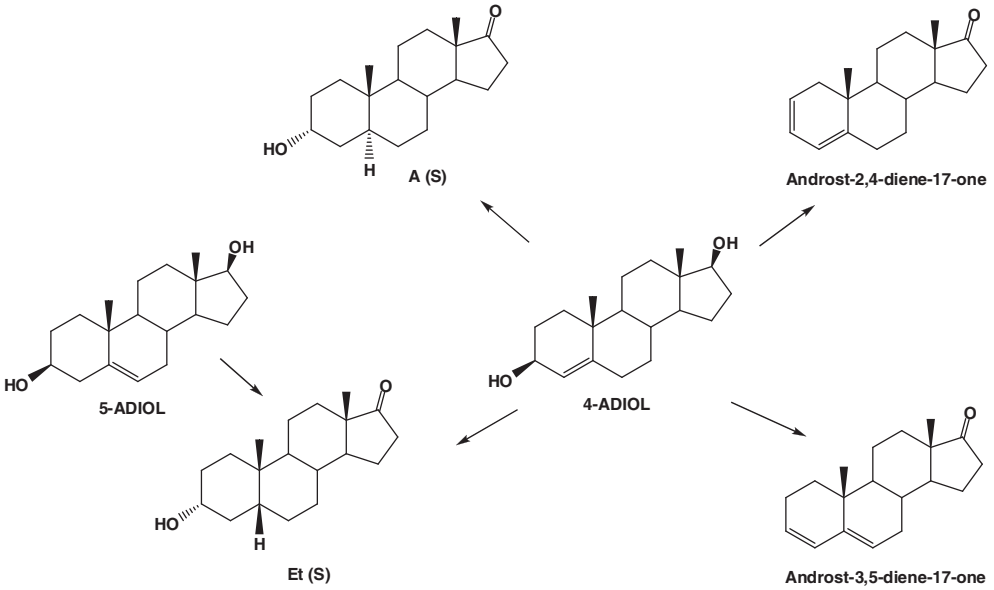

Figure 4. Biomarkers of 4-ADIOL and 5-ADIOL misuse.[16,71,94]

the scope of steroid analysis from the metabolite-only domain. The potential of androst-2,4-diene-17-one and androst-3,5-diene-17-one to specifically detect 4-ADIOL abuse demonstrates that urinary hydrolysis may also be applicable to Δ^4-steroids. By possessing a 3β-hydroxyl functional group, the C_{17}-oxidized metabolite of 4-ADIOL is likely to form a sulfoconjugate. Urinary rearrangement of the androst-4-ene-3β-ol-17-one intermediate may occur following the loss of the labile sulfoconjugate under mild acidic conditions to produce the two androstdiene markers. Further work will be required to substantiate their confirmatory value by investigating $\delta^{13}C$ variation with respect to urine degradation.[83]

5-Androstenediol (5-ADIOL)

The most pronounced effect following oral 5-ADIOL administration on the glucuronide/free steroid fraction has been the alteration of the urinary A/Et and ααβ-diol/βαβ-diol values, consistent with the steroid profiles observed following DHEA administration, with an important exception being the absence of the 3α,5-cyclo marker.[16,71] The analogous cyclosteroid; 3α,5-cyclo-5α-androstane-6β, 17β-diol had been considered to be potentially diagnostic of 5-ADIOL misuse albeit with no record in the literature. No presence of this cyclosteroid was detected following 5-ADIOL administration, however,[71] leaving further work to be done to confirm whether this mechanism is possible.

Comparing the results of studies investigating 4- and 5-ADIOL shows that the alterations in steroid profiles are distinct. Screening and confirmation of 4-ADIOL abuse using GC-MS and GC-C-IRMS analysis of androst-2,4-diene and androst-3,5-diene provides specific detection of this prohormone.[94] 5-ADIOL administration resulted in more perturbed A/Et values than those observed from 4-ADIOL.[15,16,71] Screening of 5-ADIOL abuse may rest with identifying an elevated Et-G value and a A/Et ratio of less than 0.3. For confirmation, there is a considerable preference for the metabolism of administered 5-ADIOL to produce Et-S over prolonged periods of time compared to its Δ^4-analogue. GC-C-IRMS analysis has showed no change in $\delta^{13}C$ values of DHEA-S, epiA-S or A-S following 5-ADIOL administration in contrast to 4-ADIOL administration.[71] Reasoning of 5β-specific ^{13}C-depletion observed for sulfoconjugated metabolites from Δ^5-steroid precursors requires the intermediary role of sulfoconjugated steroids in endogenous steroid metabolism to be considered. This process does not appear to represent a metabolic step prior to elimination, a function primarily performed by the liver. Sulfotransferase enzymes are largely expressed in the adrenal cortex producing sulfoconjugated steroids that circulate at levels far higher than their free forms. This creates a high concentration reservoir of precursors for conversion to other sulfoconjugated and free steroids. Furthermore, the intracellular sulfoconjugation of steroids can also serve as a storage mechanism when conjugates bind to proteins.[95] This partitioning of steroid sulfoconjugates within distinct metabolic pools represents an effective mechanism for ^{13}C fractionation as has been described for DHEA. Distinctions based on $\delta^{13}C$ values of Et-S and A-S illustrate the diagnostic potential for specific confirmation of 4- and 5-ADIOL in doping control laboratories (Figure 5).[71]

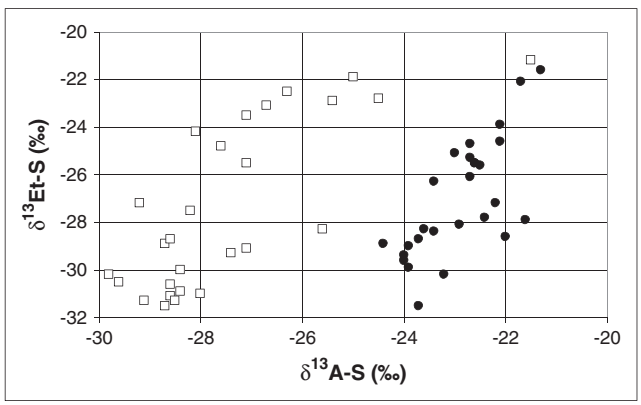

Figure 5. Discrimination of 4-ADIOL (open squares) and 5-ADIOL (closed circles) doping using $\delta^{13}C$ plots for Et-S and A-S obtained from multiple 100 mg oral administrations to a 20-year-old male volunteer.[71]

Testosterone (T): the 'holy grail' for anti-doping laboratories

The difficulty of enforcing the T/E value to identify T abuse was highlighted at the 1992 Barcelona Olympic Games, when the detection of several banned drugs in the participating athletes, namely 3 cases of stimulant medications, 2 clenbuterol positives and 3 T/E values between 6 and 10 were reported. The samples containing the stimulant drugs and clenbuterol led to sanctions being imposed on those athletes while the 3 T/E cases were referred for further study.[96] This event of course, pre-dated the use of GC-C-IRMS in doping control from which the possibility exists to confirm exogenous origin.

It is important to note, however, that stable carbon isotope ratio measurements have not been the panacea to distinguishing pharmacologically and physiologically abnormal T/E values. Effective screening criteria are required to select true doping cases for GC-C-IRMS analysis in the first instance, with a minimum number of false-negative clearances. In 2005, WADA changed the reporting threshold for elevated T/E values from 6 to 4 in order to increase the sensitivity for the detection of T misuse.[20] This required laboratories to undertake mandatory resource consuming quantification of T and E, together with GC-C-IRMS analysis, followed by longitudinal testing of individual athletes. Mareck *et al.*[97] reported only 0.4% of urine samples with T/E between 4 and 6 to deliver positive IRMS results. Most of these originated from strength sport disciplines such as weightlifting, powerlifting, wrestling, boxing and bodybuilding. Furthermore, the majority of doping cases were attributed to the male athlete population within these sports. This group also summarized the variables that need to be considered regarding the T/E as a diagnostic tool.[97] In 2010, changes concerning the management of elevated T/E cases came into effect, eliminating the need for additional collection and analysis in cases where the T/E is greater than 4 and IRMS is negative.[98]

Additional measurements have recently been proposed to complement T/E values. Goebel *et al.*[99] demonstrated suppressed luteinizing hormone (LH) concentrations in males together with T/E values greater than 4 better identify samples requiring GC-C-IRMS confirmation. Schulze *et al.*[100] have included UGT2B17 genotype information to minimize false-negative results by accounting for ethnic variations. At the same time there is a growing consensus among anti-doping laboratories, described succinctly by Kicman and Cowan,[101] that subject-based reference ranges react sensitively to pharmacological manipulations and therefore represent the most effective tool to identify T administration.[84–86] Coupled with this has been renewed interest in searching for specific markers of T misuse. Pozo *et al.*[102] characterized three novel testosterone metabolites (androsta-1,4-diene-3,17-dione, androsta-4,6-diene-3,17-dione and androsta-4,6-diene-17-ol-3-one) and a further proposed metabolite (androst-15-ene-3,17-dione) after alkaline treatment and LC-MS-MS analysis (Figure 6). These were subsequently quantified in urine samples collected 0 to 4 hours following oral administration of testosterone undecanoate to provide concentration ranges of 27.7 to 69.9 ng/ml, 73.5 to 195.4 ng/ml and 2.9 to 22.5 ng/ml for the three metabolites with available reference materials.[103] The results for androsta-4,6-diene-3,17-dione are particularly encouraging with sufficient excretion to enable future GC-C-IRMS determination of its origin as exogenous or endogenous.[104] Similarly, the administration of T in many cases increases urinary T excretion and can enable more accurate $\delta^{13}C$ measurements. GC-C-IRMS analysis is required for T concentrations greater than 200 ng/ml (SG corrected to 1.020).[20] The most effective determination of T abuse can be $\delta^{13}C$ analysis of T itself since the ^{13}C content of measured T should entirely reflect the synthetic material administered with little influence

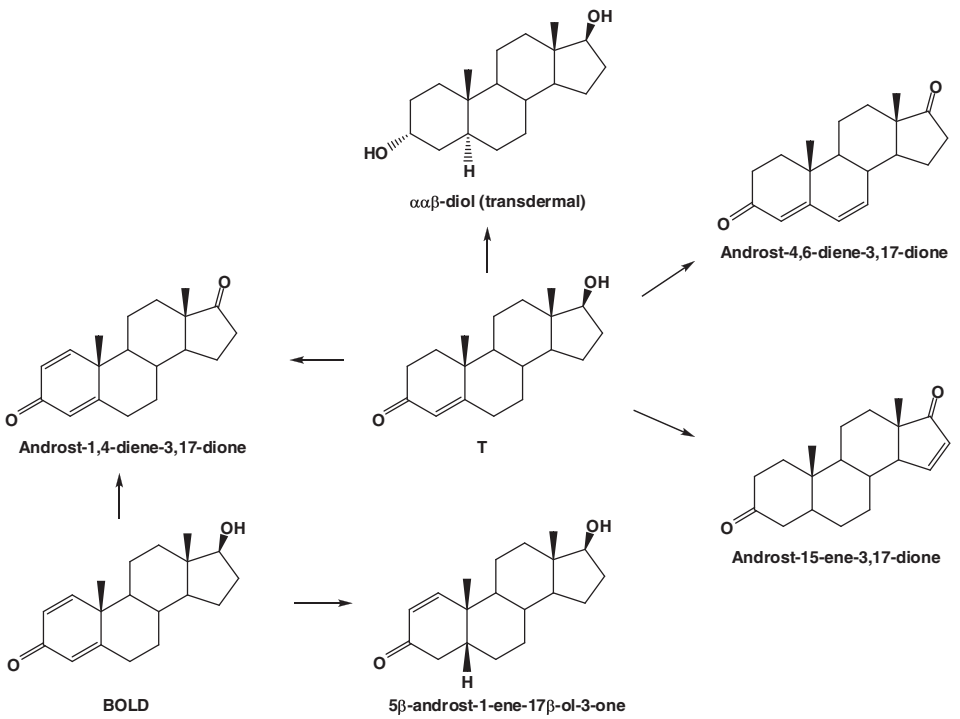

Figure 6. Biomarkers of T and BOLD misuse.[24,29,103,104,135]

of endogenous dilution due to the negative feedback mechanism governing the production of T in the body.[63]

Complementary methodologies have the potential to provide more than novel biomarkers themselves. Historically, studies investigating the alteration of the steroid profile and/or $\delta^{13}C$ values of metabolites following T administration have been limited to a single route of administration. Confounding results from discrete pharmacokinetic mechanisms, however, has then made the interpretation of findings difficult for improved anti-doping. Recent work by Van Renterghem *et al.*[70] compared steroid profile changes for different routes of T administration and different hormones – DHEA and DHT. Continuing with oral administration of T undecanoate, there is a predominant first-pass effect to produce high amounts of A-G in the first 10 h post-administration.[70] The $\delta^{13}C$ values for A-G and Et-G following similar administrations of T undecanoate, however, have been shown to display comparable trends,[105] as have ααβ-diol and βαβ-diol following intramuscular administration of T esters.[35–38] This contrasts the more distinct pattern for the relative amounts and $\delta^{13}C$ values of 5α- and 5β-reduced metabolites following transdermal application of T gels.[24,29] The $\delta^{13}C$ value of ααβ-diol reacts in a far more sensitive way due to the high 5α-reductase activity in the skin. Indeed, continuous application of T gel resulted in lower $\delta^{13}C$ values (up to 2‰) of ααβ-diol relative to the administered material, thereby indicating enzyme induction processes to be active with the excess substrate that the skin finds itself needing to metabolize.[29] Such trends raise the possibility of identifying the administration route but further studies using multiple volunteers will be required to verify this.

Dihydrotestosterone (DHT)

The 1990s saw doping practices attempt to circumvent T/E thresholds with the administration of DHT.[106] Indeed, DHT was known to be a three times more potent androgen than T due to its greater affinity for binding to the androgen receptor.[107] Considerable research was performed by anti-doping laboratories during this period to detect DHT administration.[106,108–111] These studies provided metabolic interpretations, where DHT doping was identified by ααβ-diol/βαβ-diol values greater than 1.5.[24] The sensitivity of this measurement, together with DHT/βαβ-diol, DHT/Et, DHT and DHT/T, to DHT misuse has subsequently been confirmed by Van Renterghem *et al.*[70,86] in validating the subject-based profiling strategy using Bayesian models. Alteration of the $\delta^{13}C$ values for ααβ-diol and βαβ-diol were used by Shackleton *et al.*[37] to confirm DHT administration by GC-C-IRMS. In addition, this group proposed 5α-androstane-3α,17α-diol (ααα-diol) as a route specific marker of oral DHT administration using complementary GC-MS screening and GC-C-IRMS confirmation. The C_{17}-epimerization of orally administered DHT substrate was unexpected and not observed from intramuscular or transdermal administrations.[37]

Epitestosterone (E)

Although E possesses no anabolic effect, it can be potentially misused by athletes via co-administration to mask T doping by maintaining or lowering the T/E.[112] Shackleton *et al.*[37] investigated metabolic response to E administration and found a majority excreted as unchanged drug due to the relatively low activity of 17α-hydroxysteroid dehydrogenase in humans to produce ADIONE and subsequent A and Et. The conventional C_5-reduction and subsequent C_3-hydroxylation processes were shown to produce androstanediol metabolites (ααα-diol and 5β-androstane-3α,17α-diol) with low ^{13}C content.[37] GC-MS monitoring of these metabolites is easily achieved due to their expectedly low levels (0.7 to 54.0 ng/ml for ααα-diol).[113] GC-C-IRMS confirmation would also be instigated by urinary concentrations of E (corrected for SG of 1.020) greater than 200 ng/ml.[20] Piper *et al.*[113] proposed a complementary approach using urinary concentration thresholds together with $\delta^{13}C$ values of ααα-diol and βαα-diol to more than double the post-administration detection period of E misuse. The significance of this was highlighted by the minimum $\delta^{13}C$ value of E (−31.8‰) not reaching that of the administered substrate (−33.2‰). Incomplete suppression of endogenous E production is contrary to conventional negative-feedback mechanisms governing endogenous steroid pharmacology and may therefore limit retrospectivity in terms of detection periods if urinary concentrations and $\delta^{13}C$ values are viewed in isolation.

Designer prohormones

In the past 10 years a new class of endogenous steroid analogues have been marketed as supplements and aromatase inhibitors. These compounds are considered to be naturally occurring in varying but low levels and could potentially be misused with the intention of pharmacological manipulation.

7oxo-DHEA

The first suggestion that 7oxo-dehydroepiandrosterone (7oxo-DHEA; androst-5-ene-3β-ol-7,17-dione) was excreted in human urine came from the work of Lieberman *et al.*[114] and Fukushima *et al.*[115] who isolated and characterized the free steroid after acid hydrolysis of urine. Its origin as an adrenal steroid is indicated by the fact that the compound is excreted in excessive amounts in the urine of patients with adrenal pathological conditions.[116,117] 7oxo-DHEA is distributed via the internet for its anecdotal anti-ageing effects and fat-reducing or 'thermogenic' properties. 7oxo-DHEA has been found to be metabolized to the C_7-hydroxyl isomers of androstenedione, epiandrosterone and androsterone, together with 7oxo-A.[82,118] Complementary GC-MS/GC-C-IRMS analysis of these biomarkers, together with 5ζ-androst-3,7,17-triones may enable anti-doping laboratories to confirm administration of 7oxo-DHEA (Figure 2).

4OH-ADIONE (Formestane)

Androst-4-en-ol-3,17-dione (4OH-ADIONE) is a second generation, irreversible aromatase inhibitor used as a treatment for breast cancer in post-menopausal women.[119,120] 4OH-ADIONE has been described in this review as a biomarker of ADIONE administration but is itself prohibited by WADA as a hormone antagonist and modulator.[98] The concern for anti-doping is that administration of 4OH-ADIONE may result in higher systemic levels of T due to reduced conversion of T to estrogens. Kohler *et al.*[121] investigated the metabolism of 4OH-ADIONE and its metabolite 4OH-T – prohibited by WADA as an anabolic agent – to propose 5α-androstane-3β,4α-diol-17-one as a long-term metabolite suitable for GC-MS screening (Figure 3). It was detected for up to 90 hours following oral (100 mg) administrations in both glucuronide and sulfoconjugated fractions isolated from urine.

Complementary GC-C-IRMS confirmation of surreptitious 4OH-ADIONE and/or 4OH-T misuse has recently been published.[91]

6oxo-ADIONE

Androst-4-ene-3,6,17-trione (6oxo-ADIONE) is another aromatase inhibitor, marketed as a cure for gynecomastia. It has been extensively studied by Van Eenoo and co-workers at the Ghent anti-doping laboratory in Belgium as part of their effort to identify novel hydroxylated metabolites.[82,118,122,123] GC-MS and LC-MS steroid profiling of 6oxo-ADIONE administration studies has reported 6αOH-ADIONE and 6αOH-T as major metabolites excreted primarily as their glucuronide conjugates.[122,123] In addition, 6βOH-A and 6βOH-Et were identified to be markers of 6oxo-ADIONE misuse for up to 30 h post-administration (Figure 3).[82] Similarly for the urinary metabolites of 7oxo-DHEA and 4OH-ADIONE, it is expected that this can be extended to $\delta^{13}C$ analysis of 6αOH-ADIONE and/or 6αOH-T for confirmation.

11oxo-ADIONE

Adrenosterone (androst-4-ene-3,11,17-trione; 11oxo-ADIONE) is another functionalized androstenedione derivative found in prohormone dietary supplements. Ten metabolites were reported by Brooker *et al.*[124] within 24 h of oral 11oxo-ADIONE administration. A complementary strategy was proposed that uses the concentration of 11βOH-A (> 10,000 ng/ml) and its ratio to 11βOH-Et (>20) to identify samples requiring GC-C-IRMS analysis of 11βOH-A. In addition, 11oxo-βαβ-diol was found to be specific to 11oxo-ADIONE administration, thereby distinguishing it from cortisol/cortisone manipulation.

19-nor steroids

Nandrolone (19-nortestosterone; estr-4-ene-17β-ol-3-one) and other 19-norsteroids administered orally are metabolized to form 19-norandrosterone (19-NA; 5α-estrane-3α-ol-17-one), 19-noretiocholanolone (19-NE; 5β-estrane-3α-ol-17-one) and 19-norepiandrosterone (19-NEA; 5α-estrane-3β-ol-17-one).[15,16] The first two are predominantly excreted in urine as glucuronide conjugates, while the latter is found exclusively as its sulfoconjugate. Phase II metabolic comparisons were made by Tseng *et al.*[125] and Torrado *et al.*[126] that showed sulfoconjugate amounts to be higher than glucuronides at later excretion times consistent with slower elimination from the kidneys. This supported the findings of studies reviewed previously relating to DHEA and 5-ADIOL.[16,48,71,81] The potential for discrete glucuronide and sulfoconjugate excretion of 19-nor steroids to provide evidence of origin was investigated by Guay *et al.*[127] and Strahm *et al.*[128] who concluded that IRMS was required to confirm any abnormal metabolic flux observations. On a pharmacokinetic level, these studies demonstrated that sulfoconjugation is predominant at low substrate concentration whereas glucuronidation is the major conjugation process at high substrate levels because of saturation kinetics affecting the sulfation reaction.[129] The lower concentrations of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) co-factor in the liver with respect to uridine diphosphate-glucuronic acid (UDPGA) may result in a more rapid consumption of PAPS and consequently the efficiency of sulfotransferase enzymes will be affected during the early periods of excretion.[130,131]

The potential for 19-NA to be present in the urine of female athletes, as a by-product of aromatization resulting in an increased production of estrogens during pregnancy or ovulation, led to a concerted effort by the Cologne anti-doping laboratory to elucidate the endogenous or exogenous origin of 19-NA.[132] The challenge to measure $\delta^{13}C$ values from as little as 2 ng/ml urinary 19-NA was met with the development of dual HPLC clean-up procedures utilizing both normal and reversed-phase chemistries.[132] This methodology was simplified by de la Torre *et al.*[133] to investigate the measurement of $\delta^{13}C$ values for urinary 19-NA arising from pregnant females with concentrations down to 1.6 ng/ml. These $\delta^{13}C$ values were compared to those obtained from a single oral 19nor-5-androstenediol (19N5-ADIOL; estr-5-ene-3β,17β-diol) administration study that recorded $\Delta\delta^{13}C$ values up to 8.5‰ using A-G as the ERC.[133] This work enabled confirmation of adverse analytical findings in routine anti-doping samples displaying $\Delta\delta^{13}C$ values between 6.7‰ and 12.1‰.[133] In addition, a survey of commercially available 19-NA precursor compounds was performed to provide a $\delta^{13}C$ range of -26.3 ± 0.3‰ to -30.9 ± 0.3‰, the latter belonging to the 19N5-ADIOL product used for the administration study.[133]

On the topic of biomarkers, the work of Lévesque *et al.*[134] represents an alternative strategy for identifying novel targets for doping control. An *in vitro* study of 19nor-ADIONE offered the ability to work with cleaner matrices, optimize metabolite formation by adjusting experimental conditions, propose new metabolic routes via the identification of intermediates, and produce reference materials of novel metabolites in an efficient manner. There is little doubt that *in vitro* systems will be used more frequently to validate combinatorial models and investigate ^{13}C fraction patterns of substrates and co-factors in the future.

Boldenone

Boldenone (BOLD; androst-1,4-diene-17β-ol-3-one) is another steroid for which questions regarding endogenous levels have been raised. Using the results of metabolic studies conducted by Schänzer and Donike,[135] Piper *et al.*[136] validated a method to measure the $\delta^{13}C$ values of BOLD and/or its major metabolite 5β-androst-1-ene-17β-ol-3-one at urinary concentrations of between 2 and 50 ng/ml. Translation of this method into practice found 11 out of 23 boldenone findings to be inconsistent with synthetic boldenone administration.[136] An additional metabolite to be considered in future determinations is Androst-1,4-diene-3,17-dione (Figure 6). GC-C-IRMS analysis of samples with low urinary BOLD was recommended to avoid false-positive results. The $\delta^{13}C$ values of six commercially available preparations containing BOLD were determined, five of which displayed expectedly low ^{13}C content (-26.3‰ to -30.3‰). One equine veterinary product from 1985, fortunately no longer available, recorded a $\delta^{13}C$ value of -22.7‰.

Glucocorticosteroids

Stable carbon isotope ratio analysis has been used to distinguish naturally occurring glucocorticosteroids, namely hydrocortisone and cortisone, from their synthetic analogues.[137–139] The measurement of GC-amenable tetrahydrocortisol and tetrahydrocortisone oxidation products was extended by Buisson *et al.*[140] to include an ERC and therefore present data as $\Delta\delta^{13}C$ values. Interestingly the ERC's proposed for this purpose were the oxidation products

Table 1. Urinary steroid markers for doping control

Target steroid	Biomarker[(s)]	GC-MS screening limit	GC-C-IRMS confirmation	References
DHEA	DHEA[(G)]	100 ng/ml*	Y	[20]
	3α,5-cyclo	100 ng/ml*	Y	[24,80–82]
	7βOH-DHEA	126 ng/ml*	Y	[70,86]
	16αOH-DHEA	37 ng/ml*		[48]
	epiA[(S)]	673 ng/ml*		
7oxo-DHEA	7αOH-epiA, 7βOH-epiA, 7oxo-DHEA, 7oxo-A, 5α/β-androstane-3,7,17-triones	-		[82,118]
ADIONE	6αOH-ADIONE, 6βOH-A, 6βOH-Et	-	Y	[88]
	4OH-ADIONE	40 ng/ml*		[89–92]
4OH-ADIONE	4OH-ADIONE,	50 ng/ml*	Y	[91,98]
	5α-androstane-3β,4α-diol-17-one	-		[121]
6oxo-ADIONE	6αOH-ADIONE, 6αOH-T,6βOH-A, 6βOH-Et	-		[82,122,123]
11oxo-ADIONE	11βOH-A	10,000 ng/ml*	Y	[124]
	11βOH-A/11βOH-Et	20	Y	
	11oxo-βαβ-diol			
4-ADIOL	Androst-2,4-diene-17-one	-	Y	[94]
	Androst-3,5-diene-17-one		Y	
5-ADIOL	A/Et	<0.3		[16,71]
T	T	200 ng/ml*	Y	[20]
	T/E	4	Y	[97,98]
	LH	<2 IU/L*		[99]
	Androsta-4,6-diene-3,17-dione	56 ng/ml*		[103,104]
	Androst-1,4-diene-3,17-dione	1 ng/ml*		[24,29]
	Androst-15-ene-3,17-dione	3 ng/ml*		
	ααβ-diol/βαβ-diol (transdermal administration)	>1.5		
DHT	ααβ-diol/βαβ-diol (transdermal administration)	>1.5		[24]
	ααα-diol (oral administration)	-		[37]
	DHT	21 ng/ml*		[70,86]
E	E	200 ng/ml*	Y	[20]
	ααα-diol, βαα-diol			[37,113]
19nor steroids	19-NA	2 ng/ml*	Y	[20]
	19-NE, 19-ENA			[15,16]
BOLD	BOLD, Androst-1,4-diene-3,17-dione 5β-androst-1-ene-17β-ol-3-one	2 ng/ml*	Y	[135]

* Concentration corrected to a specific gravity of 1.020 g/ml[20]
[(G)] = measured from free/glucuronide fraction,
[(S)] = measured from sulfoconjugate fraction

of conventional target analytes; A (as 5α-androstane-3,17-dione) and Et (as 5β-androstane-3,17-dione). The reported isomer-related differences in absolute $\delta^{13}C$ values compared well to previous studies investigating kinetic isotope effects.[72,77]

Screening with high resolution-accurate mass technologies

Analytical platforms using the latest generation mass spectrometers with high resolving power complemented with advanced pattern-recognition software are ideal for implementing a combinatorial approach to anti-doping. Recent coupling of ultra-high pressure ion trap mass spectrometry (UHPLC) to such platforms has demonstrated the ability of untargeted steroidomic analysis measuring intact steroid glucuronide and sulfoconjugates to identify samples originating from oral testosterone undecanoate administration.[141,142] One important conclusion of this work was to emphasize the need to monitor both glucuronide and sulfoconjugates of steroids for inclusion into the *athlete steroidal passport*.[143] In addition, the use of complementary LC-based screening methods will enable less volatile analytes such as glucocorticosteroids to be assessed.

Conclusion

The objective to maximize the detection of synthetic steroid doping violations while minimizing the additional resources required from stakeholders can be achieved by complementary strategies implementing advanced steroid profiling and confirmatory GC-C-IRMS analysis. A paradigm shift is taking place in doping

analysis towards subject-based profiling of endogenous steroids. This was initially developed for longitudinal monitoring of the T/E value but has now been extended to examine the acute effects of prohormone and metabolite misuse that do not necessarily alter the T/E. The application of biomarkers has increased the sensitivity and selectivity of screening methods, and the ability of GC-C-IRMS to confirm these specific administrations. Table 1 summarizes the biomarkers currently considered in doping analysis. Beyond analyzing known steroid metabolites, these strategies can take advantage of metabolic or chemical transformations specific to endogenous steroid precursors. The basis for this proposal is to use all of the available information relating to an athlete's metabolism to effectively confirm the illegal administration of synthetic steroid copies.

Acknowledgements

The authors acknowledge the assistance of Associate Professor Robert Weatherby from the Department of Exercise Science at Southern Cross University for providing some of the initial questions concerning steroid pharmacology from which complementary investigations were pursued at the Sydney anti-doping laboratory. Many of the technical improvements for GC-C-IRMS in anti-doping have been assisted by Dr Tom Brenna from the Department of Nutrition Research at Cornell University, Ithaca, NY, USA. Professor Mario Thevis and Ulrich Flenker from the Manfred Donike Institute in Cologne, Germany have each assisted many of the research groups engaged in this type of analysis for anti-doping. It is important to acknowledge that much needed funding for understanding stable isotope ratio measurements and applying knowledge to GC-C-IRMS analysis in anti-doping has been provided over the past decade by organizations including, but not limited to; the World Anti-Doping Agency (WADA), United States Anti-Doping Agency (USADA), United States Partnership for Clean Competition, the Federal Ministry of the German Interior, and the Anti-Doping Research Program of the Australian Commonwealth Government.

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