Research Article

Drug Testing and Analysis

Received: 1 July 2009 Revised: 2 December 2009 Accepted: 3 December 2009 Published online in Wiley Interscience:

(www.drugtestinganalysis.com) DOI 10.1002/dta.108

Development of criteria for the detection of adrenosterone administration by gas chromatography-mass spectrometry and gas chromatography-combustion-isotope ratio mass spectrometry for doping control

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Adrenosterone (androst-4-ene-3,11,17-trione, 11-oxoandrostenedione) is an endogenous steroid hormone that has been promoted as a dietary supplement capable of reducing body fat and increasing muscle mass. It is proposed that adrenosterone may function as an inhibitor of the 11β-hydroxysteroid dehydrogenase type 1 enzyme (11β-HSD1), which is primarily responsible for reactivation of cortisol from cortisone. The urinary metabolism of adrenosterone was investigated, after a single oral administration in two male subjects, by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Substantially increased excretion of 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone, 11-oxoandrosterone and 11-oxoetiocholanolone was observed. Minor metabolites such as 3α,17β-dihydroxy-5β-androstan-11-one, 3α-hydroxyandrost-4-ene-11,17-dione and 3α,11β-dihydroxyandrost-4-en-17-one were also identified. The exogenous origin of the most abundant adrenosterone metabolites was confirmed by GC-C-IRMS according to World Anti-Doping Agency criteria. Through analysis of a reference population data set obtained from urine samples provided by elite athlete volunteers (n = 85), GC-MS doping control screening criteria are proposed:

- **11β-hydroxyandrosterone concentration greater than 10 000 ng/mL (specific gravity adjusted to 1.020)** *or*
- **11β-hydroxyandrosterone/11β-hydroxyetiocholanolone ratio greater than 20.**

Urine samples fulfilling these screening criteria may be subjected to GC-C-IRMS analysis for confirmation of adrenosterone administration.

Keywords: anabolic androgenic steroids; dietary supplements; doping control; 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1); mass spectrometry

Introduction

For more than ten years, anabolic androgenic steroid-based products have been available on the international dietary supplement market.[1,2] Although originally based on precursors of testosterone,[3,4] since the appearance of designer steroids[5–8] numerous examples of 'designer' supplements have also been identified.[9,10] These 'prohormones' have been shown to contain steroids never before marketed as legitimate therapeutic drugs.[11–15] Recently, a new class of designer prohormone has emerged – aromatase inhibitors based on functionalized androstenedione derivatives. Like the legitimate anti-cancer drugs, exemestane,[16] formestane[17] and testolactone,[18] which are on the World Anti-Doping Agency (WADA) 2009 Prohibited List,[19] these steroids are primarily promoted for their anti-estrogenic properties.[20,21] Examples include, androst-4-ene-3,6,17-trione (6-OXO),[22] 6α-methylandrost-4-ene-3,17-dione (Formadrol),[13,23,24] 6-bromoandrost-4-ene-3,17-dione (Hyperdrol)[25] and androsta-1,4,6-triene-3,17-dione (ATD).[15,23,26]

Adrenosterone (androst-4-ene-3,11,17-trione, 11-oxoandrostenedione, 11-OXO, Figure 1) is another functionalized androstenedione derivative found in prohormone dietary supplements. However, like the WADA-prohibited substances dehydroepiandrosterone, androstenedione and cortisol, it is also an endogenous steroid hormone secreted by the adrenal gland.[27,28]

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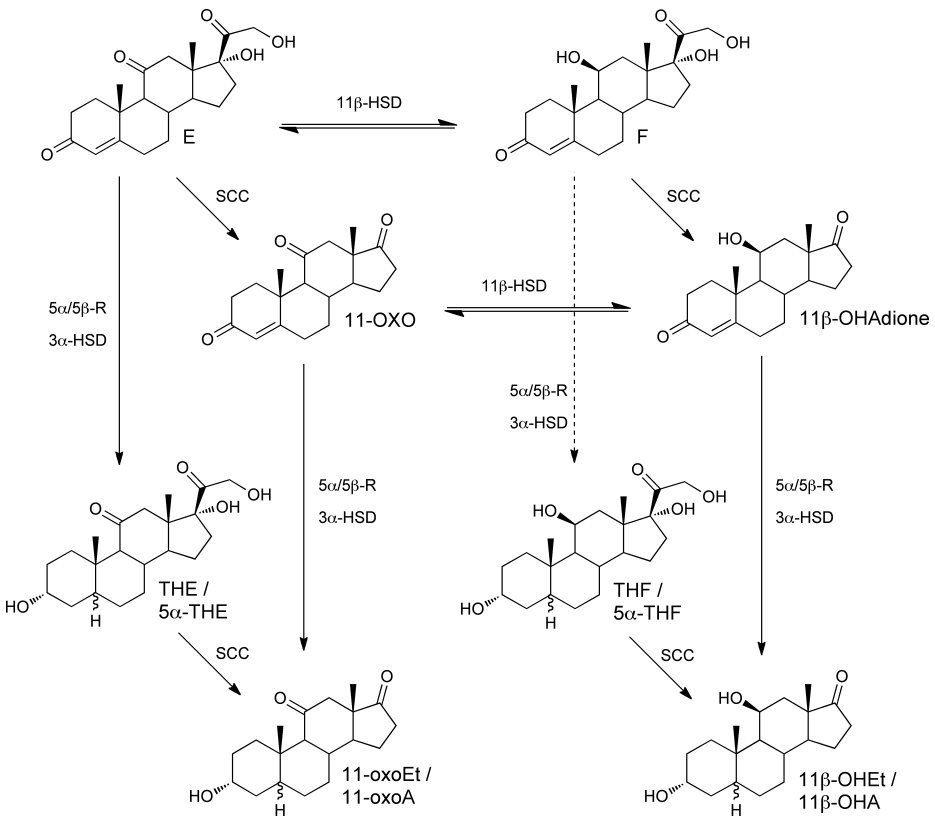

Figure 1. Adrenosterone (11-OXO) biosynthesis and metabolism. Endogenous adrenosterone may be formed by the conversion of adrenally secreted 11β-hydroxyandrostenedione (11β-OHAdione) or the peripheral side-chain cleavage the endogenous corticosteroids, cortisol (F) or cortisone (E). Likewise, the 11-oxy-C_{19} metabolites (11-oxoEt/11-oxoA and 11β-OHEt/11β-OHA) of adrenosterone and 11β-hydroxyandrostenedione may also be formed from the major corticosteroid metabolites (THE/5α-THE and THF/5α-THF).[35]
SCC = Side chain cleaving enzyme.
11β-HSD = 11β-hydroxysteroid dehydrogenase enzyme.
3α-HSD = 3α-hydroxysteroid dehydrogenase enzyme.
5α/5β-R = 5α/5β-reductase enzyme.

The adrenosterone-containing supplement is promoted as a 'selective cortisol modulator' rather than an anti-estrogen, possessing capabilities to decrease body fat and increase muscle density. A reduction in elevated cortisol may have a performance-enhancing benefit for athletes.[29,30] It has been shown that adrenosterone is a selective inhibitor of the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1),[31] which is primarily responsible for the re-activation of cortisol (F) from cortisone (E) (Figure 1).[32]

Supplementation of adrenosterone at supra-physiological doses could be expected to reduce cortisol production from cortisone significantly, by being a competitive substrate to the 11β-HSD1 enzyme present in excessive amounts. Adrenosterone and its corresponding conversion product 11β-hydroxyandrostenedione (11β-hydroxyandrost-4-ene-3,17-dione, 11β-OHAdione, Figure 1) are also considered to be androgens, although much less potent in relation to testosterone.[33,34]

Two potential biosynthetic pathways of adrenosterone exist: the conversion of adrenally secreted 11β-hydroxyandrostenedione by 11β-HSD and the peripheral side-chain cleavage of the endogenous corticosteroids cortisol (11β,17,21-trihydroxypregn-4-ene-3,20-dione, F) and cortisone (17,21-dihydroxypregn-4-ene-3,11,20-trione, E) (Figure 1).[35] Four major urinary metabolites of adrenosterone and 11β-hydroxyandrostenedione have been identified: 11β-hydroxyetiocholanolone (3α,11β-dihydroxy-5β-androstan-17-one, 11β-OHEt), 11-oxoetiocholanolone (3α-hydroxy-5β-androstane-11,17-dione, 11-oxoEt), 11β-hydroxyandrosterone (3α,11β-dihydroxy-5α-androstan-17-one, 11β-OHA) and 11-oxoandrosterone (3α-hydroxy-5α-androstane-11,17-dione, 11-oxoA).[36–40]

These 11-oxygenated C_{19} (11-oxy-C_{19}) metabolites are routinely assessed within urinary steroid profiling assays for clinical[41] and doping-control purposes.[42,43] Pronounced changes in the urinary steroid metabolic profile can indicate particular disease states,[44] genetic conditions[45–47] or the exogenous administration of endogenous anabolic androgenic steroids such as dehydroepiandrosterone.[48–52] They have also been utilized as 'endogenous reference compounds' (ERC)[53,54] in carbon isotope ratio tests for the confirmation of endogenous anabolic androgenic steroid abuse in doping control.[55–57] The WADA has set a threshold difference between ERC and target compound (TC) of greater than 3‰ as being consistent with the administration of a steroid.[58] Recently, the 11-oxy-C_{19} metabolites' primary role as ERC has been reversed, acting as 'TC' in the confirmation of the administration of endogenous corticosteroids (Figure 1).[59,60] In a similar way, it is anticipated that routine GC-C-IRMS methods that utilize 11β-OHEt, 11β-OHA or 11-oxoEt as ERC could be used to confirm adrenosterone administration.

Two separate administration studies of adrenosterone were conducted (DSHS, Cologne, Germany and ASDTL, Sydney, Australia)

and the resulting urine samples were analysed by each laboratory's routine GC-MS[50,61] and GC-C-IRMS[55,57] methods. Comparison of these data with previously obtained reference population data enabled anti-doping screening criteria for adrenosterone abuse to be proposed. In addition, the identification of novel metabolites has highlighted the potential for more sensitive, compound-specific markers of adrenosterone abuse to be implemented into routine steroid profiling.

Experimental

Reference materials

All steroid reference materials utilized for analyses were obtained from Steraloids (Newport, RI, USA), Sigma Chemical Co. (St Louis, MO, USA) or Chemical Reference Materials, National Measurement Institute (Pymble, Australia). 5α-Androstan-3β-ol (5α-ol, CU/USADA 30-1) was a gift from the Division of Nutritional Sciences, Cornell University (Ithaca, NY, USA).

Chemicals and reagents

β-Glucuronidase enzyme from *Escherichia coli* K12 was obtained from Roche Diagnostics (Mannheim, Germany). Various laboratory chemicals and reagents were obtained from Ajax Finechem (Australia) and Pronalys Chemicals (Biolab, Australia). All solvents were of HPLC grade and were obtained from Merck (Darmstadt, Germany). Water was obtained using a Millipore filtration system (Bedford, MA, USA). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Chem. Fabrik Karl Bucher (Waldstetten, Germany). K-selectride® was obtained from Sigma-Aldrich (USA).

Dietary supplement

A dietary supplement, labelled as containing 75 mg of adrenosterone per capsule, was obtained from the major supplier of this type of product (11-OXO™, Ergopharm Performance Nutrition, Proviant Technologies Inc, IL, USA). A maximum recommended dose of three capsules, twice a day, for up to eight weeks was suggested.

For confirmation of the steroidal ingredients of the supplement, extraction and analysis of the capsules content was undertaken.[24,50] The homogenized content of one capsule was shaken with methanol (5 mL, 30 mins, rotary mixer). After centrifugation (20 mins, 1000 *g*), the organic layer was transferred to a clean test tube and evaporated to dryness under nitrogen (40 °C, 25 psi). Portions of this extract were then further prepared for GC-MS and GC-C-IRMS analysis.

Administration studies

Two healthy male subjects (Subject S, Subject C) completed an administration study using one capsule each of the 11-OXO™ supplement (75 mg adrenosterone). Appropriate ethics approval was obtained and no special diet or exercise regime was maintained during the study period. Spot urine samples were collected before and after administration for up to 48 hours (Subject S – 13 samples, Subject C – 14 samples). Urine samples were measured for pH and specific gravity and stored at −20 °C until analysis.

Reference population

The reference population consisted of urine samples collected from 85 elite athlete volunteers, with written informed consent and ethics approval.[57] Samples originated from Australia (AUS, $n = 29$, 16 male, 13 female, average age 24 years), New Zealand (NWZ, $n = 33$, 25 male, eight female, average age 20 years) and Malaysia (MAL, $n = 23$, 22 male, one female, average age 23 years). After collection, samples were measured for pH and specific gravity and stored at −20 °C until analysis.

Sample preparation and analysis

Gas chromatography – mass spectrometry

The measurement of steroid concentrations utilized routine validated steroid profiling procedures[50,61] established for the screening of all urine samples submitted for doping control,[58] common to all WADA accredited laboratories.[62] In general, a surrogate solution containing appropriate concentrations of d_3-testosterone and d_4-androsterone glucuronide was added to an aliquot of urine. After addition of phosphate buffer and β-glucuronidase enzyme the sample was incubated. The deconjugated steroids were isolated from the urine matrix with either liquid-liquid[61] or solid phase extraction.[50] 17α-Methyltestosterone volumetric internal standard was added to the final extract, then it was dried and derivatized with TMSI reagent (MSTFA/NH_4I/ethanethiol, 1000 : 2:3, v/w/v, 55 µL, 60 °C, 15 – 20 min) to form trimethylsilyl derivatives. Analysis of the MSTFA derivatized urine extracts for steroid profiling was performed on a single quadrupole GC-MS in single ion monitoring (SIM) and full scan (*m/z* 50 – 400) modes as previously reported.[50,61] The concentrations determined for the analytes 11β-OHA and 11β-OHEt were estimates provided from one-point calibration curves – a full measurement uncertainty budget has not been completed at this time.

Gas chromatography-combustion-isotope ratio mass spectrometry

Selected urine samples were prepared by previously reported GC-C-IRMS confirmation methods.[55,57] Steroid glucuronides were isolated from the urine (4 – 20 mL) using solid phase extraction. After enzymatic hydrolysis with β-glucuronidase the resulting deconjugated steroids were isolated by liquid-liquid extraction (K_2CO_3, pH 9, *tert*-butyl-methyl ether). The combined extract was fortified with β-trenbolone retention time marker, dried and reconstituted in methanol for purification by semi-preparative high performance liquid chromatography (HPLC) on a reverse-phase C18 column with automated fraction collection.[55,57] For this study, two major fractions were collected. Fraction 1 (11-oxy-C_{19} metabolites) was collected between 12.9 – 14.5 min and Fraction 2 (Et and A) was collected between 19.5 and 21.6 min, relative to β-trenbolone at 12.1 min. A high concentration standard of these analytes was run at the beginning of each batch to check collection windows. Chromatography was monitored at 210 nm and 345 nm (λ_{max} of β-trenbolone). After collection, each fraction was supplemented with internal standard (5α-androstan-3β-ol) thoroughly dried, and reconstituted in an appropriate volume of solvent (cyclohexane[55] or ethyl acetate[57]). As previously reported, samples were screened by full-scan GC-MS (*m/z* 50 – 400) prior to GC-C-IRMS analysis to confirm analyte identity, establish peak purity and to estimate the analyte concentration.[57,63] After appropriate adjustment of the extract volume, the sample was analysed by GC-C-IRMS as previously described.[55,57]

Table 1. GC-MS retention times of reference materials of adrenosterone and metabolites, underivatized and TMS derivatized

	RT (min)		Relative retention	
Analyte	*Underivatized*	*TMS*	*Underivatized (5α-ol)*	*TMS (d_3-T)*
5α-androstan-3β-ol	9.06	–	1.000	–
d_3-testosterone	–	11.81	–	1.000
11-OXO	20.47	13.20	2.259	1.118
11β-OHA	20.00	12.22	2.207	1.035
11β-OHEt	18.83	12.47	2.078	1.056
11-oxoA	17.33	10.61	1.912	0.898
11-oxoEt	16.23	10.97	1.792	0.929
11β-OHAdione	24.62	14.87	2.717	1.259
11-oxoT	22.02	13.92	2.431	1.179
11β-OHT	24.77	15.35	2.734	1.300
11-oxo-diol	17.61	11.67	1.943	0.988
Metabolite **I**	16.60	*11.81**	1.832	*0.898**
Metabolite **II**	19.20	*13.25**	2.120	*1.008**

* Retention times from GC-MS analysis of synthesis products.

Synthesis of reference materials

*3ξ-Hydroxyandrost-4-ene-11,17-dione (**I**) and 3ξ,11β-dihydroxyandrost-4-en-17-dione (**II**)*

For metabolite identification, adrenosterone or 11β-hydroxyandrostenedione were reduced on a mg scale in 1 mL of absolute tetrahydrofuran with 10% molar excess of K-selectride solution. After 1 h at ambient temperature, 1 mL of aqueous ammonium chloride solution and 6 mL of TBME were added and the resulting isomers **(Ia** and **Ib)** and **(IIa** and**IIb)** respectively were extracted. The products were analysed by GC-MS and their mass spectra and retention times were compared to the urinary metabolites of adrenosterone.

Results

Analysis of dietary supplement

Gas chromatography – mass spectrometry analysis of capsule extracts and steroid reference materials confirmed the presence of adrenosterone in the 11-OXO™ dietary supplement (GC-MS retention times in Table 1). No major steroidal impurities were identified. Gas chromatography – combustion-isotope ratio mass spectrometry analysis of underivatized capsule extracts determined the $\delta^{13}C$ of the adrenosterone in the capsules as -30.4 ± 0.5 ‰ (n = 7, 1 s.d.). For comparison, the $\delta^{13}C$ of an adrenosterone reference material obtained from Steraloids was -29.0 ± 0.1 ‰ (n = 7, 1 s.d.). Significantly, adrenosterone has been identified by GC-MS in unlabelled capsules that were seized by the Australian Customs Service and submitted to the Australian Forensic Drug Laboratory of the National Measurement Institute for routine analysis.[64] In this instance, the $\delta^{13}C$ value of the recovered adrenosterone was -29.8 ± 0.2 ‰ (n = 3, 1 s.d.) by EA-IRMS.

Gas chromatography – mass spectrometry steroid profiling of administration studies

Routine steroid profiling for both administration studies revealed a substantial increase in the excretion of the adrenosterone metabolites 11β-OHA and 11β-OHEt after the ingestion of the 11-OXO™ supplement. Significant dilution of immediate post-administration study samples was required to provide baseline chromatographic separation of 11β-OHA and 11β-OHEt, with the resulting concentrations adjusted by the appropriate dilution factor. Peak excretion of 11β-OHA occurred within six hours, reaching concentrations in excess of 50 µg/mL (Figure 2). However, concentrations returned to pre-administration levels by 24 hours. Both of these findings are an indication of the short half-life of adrenosterone. Additionally, a relatively smaller increase in the excretion of 11β-OHEt was observed, leading to an elevated 11β-OHA/11β-OHEt ratio. For both subjects, the maximum ratio was observed within the first six hours after administration (45.7 and 36.7 for Subjects S and C respectively) and returned to pre-administration levels before 24 hours.

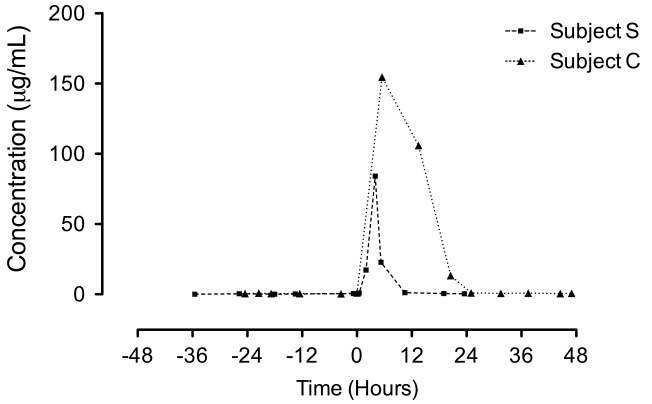

Figure 2. Excretion profile of estimated 11β-OHA concentration (µg/mL, specific gravity adjusted to 1.020) after 11-OXO™ administration.

Gas chromatography – mass spectrometry full scan analysis of administration studies

The other major adrenosterone metabolites 11-oxoEt and 11-oxoA, were identified in GC-MS full-scan analysis of both derivatized and underivatized administration study extracts. The maximum concentration of 11-oxoEt found was estimated in the very high µg/mL levels as a significantly greater than tenfold dilution of the urine extract was required to obtain acceptable chromatography of the analyte, derivatized or underivatized. The elevated levels of 11-oxoA observed might be considered indicative of adrenosterone administration as this analyte is not generally found at significant concentrations in routine urinary 11-oxy-C_{19} metabolite analysis.[35,57] A number of reduced metabolites of adrenosterone were identified by using appropriate reference materials for comparison including 11β-hydroxyandrostenedione, 11-oxotestosterone (17β-hydroxyandrost-4-ene-3,11-dione, 11-oxoT) and 11β-hydroxytestosterone (11β,17β-dihydroxyandrost-4-en-3-one, 11β-OHT) (Table 1). The increased excretion of these analytes after administration highlights the capability of adrenosterone to be converted into products reported in literature to show increased anabolic properties.[33,34]

Further investigation revealed the presence of other potential metabolites. 3α,17β-Dihydroxy-5β-androstan-11-one (11-oxo-diol) was initially identified as an underivatized compound by GC-MS analysis of GC-C-IRMS screening extracts with reference to library spectra.[65] Retention time and mass spectra comparison with reference material confirmed the presence of 11-oxo-diol (Table 1, Figure 3). Subsequent steroid profiling

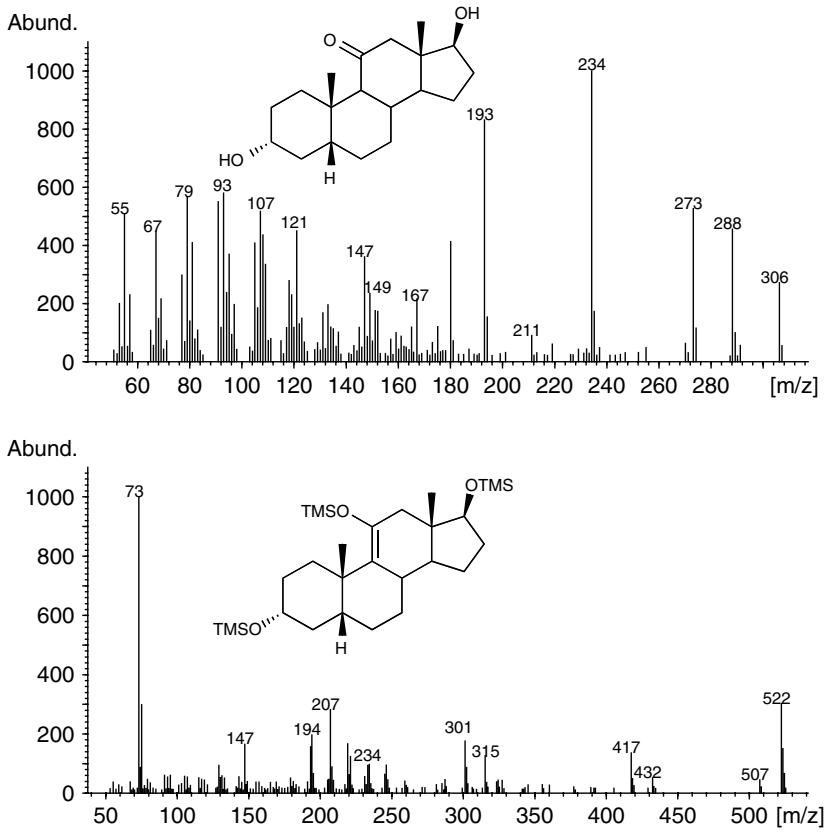

Figure 3. Mass spectra (EI) of 3α,17β-dihydroxy-5β-androstan-11-one (11-oxo-diol): (1) underivatized, $M^+ = 306$; (2) tris-TMS derivative, $M^+ = 522$.

quantification of the Subject S study as the tris-TMS derivative (SIM, $M^+ = 522$, $RT_{tris-TMS} = 11.67$ min, $RRT_{d3-TIS} = 0.988$, Figure 3) revealed its prolonged excretion relative to the major adrenosterone metabolites – the concentration at 19 hours after administration was 100 ng/mL, approximately 20 times the average pre-administration concentration (5 ng/mL). Two partially reduced metabolites were also identified. In underivatized extracts, Metabolite **I** eluted directly after 11-oxoEt with $[M]^+ = 302$ ($RT_{underivatized} = 16.60$ min, $RRT_{5\alpha-ol\ IS} = 1.832$). Metabolite **II** eluted directly after 11β-OHEt with $[M]^+ = 304$ ($RT_{underivatized} = 19.20$ min, $RRT_{5\alpha-ol\ IS} = 2.120$). The proposed structures for metabolites **I** and **II** are 3α-hydroxyandrost-4-ene-11,17-dione and 3α,11β-dihydroxyandrost-4-en-17-one respectively as these compounds have previously been reported as significant metabolites of adrenosterone in a hypothyroid patient with impaired 5α-reductase activity.[39]

Synthesis of reference materials

*3ξ-Hydroxyandrost-4-ene-11,17-dione (**I**) and 3ξ,11β-dihydroxyandrost-4-en-17-one (**II**)*

The reduction of adrenosterone with K-selectride yielded the two isomeric 3α- (**Ia**) and 3β-hydroxy (**Ib**) analogues ($RT_{Ia,tris-TMS} = 11.81$ min, $RT_{Ib,tris-TMS} = 13.15$ min). Analogously, 11β-hydroxyandrostenedione was reduced to its corresponding 3ξ-hydroxy steroids ($RT_{IIa,tris-TMS} = 13.25$ min, $RT_{IIb,tris-TMS} = 15.06$ min). In accordance with previous investigations[66,67] this reduction of 3-oxo-4-ene steroids mainly results in 3β-hydroxy isomers, which show longer retention times than the 3α-hydroxy isomers. Comparison of the GC-MS retention times (Table 1) and spectra (Figure 4) of the 3α-isomers **Ia** and **IIa** in the respective mixtures with metabolite **I** and **II** of the adrenosterone administration urines matched and thus confirmed the proposed structures.

Gas chromatography-combustion-isotope ratio mass spectrometry analysis of administration studies

GC-C-IRMS studies focused on the analysis of one major adrenosterone metabolite, 11β-OHA and the testosterone metabolite, androsterone (3α-hydroxy-5α-androstan-17-one, A).[56] As expected, for both subjects the $\delta^{13}C$ values of 11β-OHA approached that of the administered adrenosterone substrate (Figure 5). Subject C displayed more prolonged depletion as well as greater variability of $\delta^{13}C$ values of 11β-OHA in post-administration samples relative to Subject S.

In contrast, A was not influenced by the administration of adrenosterone (Figure 5). In this instance, A can be used as an endogenous reference compound for comparison to 11β-OHA. To maintain accepted protocol, the isotopic difference between the metabolites ($\Delta\delta^{13}C$) was calculated as per WADA criteria,[58] with 11β-OHA as 'ERC' and A as 'TC'.[56] As expected then, the $\Delta\delta^{13}C$ marker 11β-OHA – A *decreased* significantly after adrenosterone administration for both subjects (Figure 6). The $\Delta\delta^{13}C$ fell below −6‰ and, again, Subject C displayed more variable depletion, for a longer period than Subject S. Despite this, for both subjects a $\Delta\delta^{13}C$ of below −3‰ was observed for up to approximately 24 hours. It must be noted that significant $\delta^{13}C$ changes were also observed for the other 11-oxy-C_{19} metabolites, 11β-OHEt and 11-oxoEt.

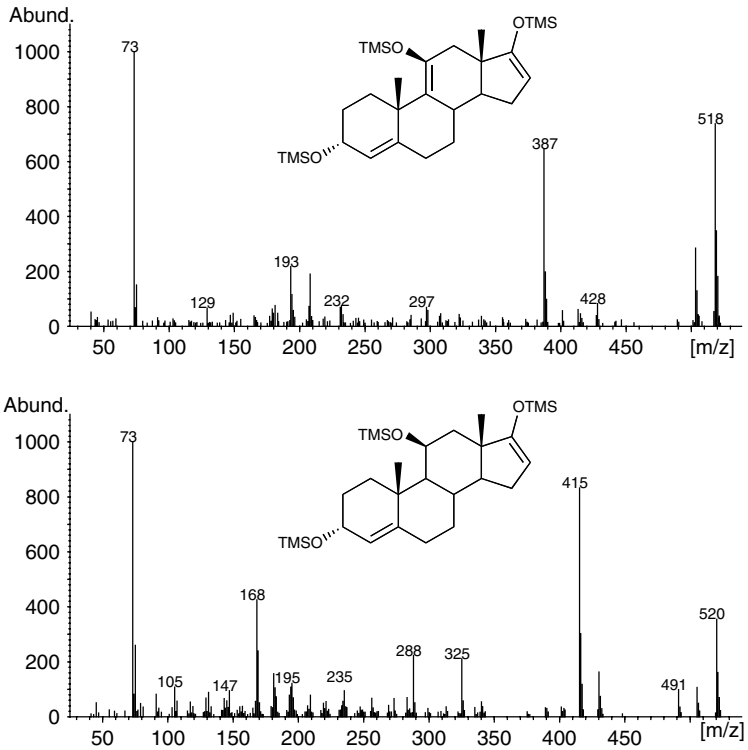

Figure 4. (1) Mass spectrum (EI) of 3α-hydroxyandrost-4-ene-11,17-dione (Ia), tris-TMS, $M^+ = 518$. (2) Mass spectrum (EI) of 3α,11β-dihydroxyandrost-4-en-17-one (IIa), tris-TMS, $M^+ = 520$.

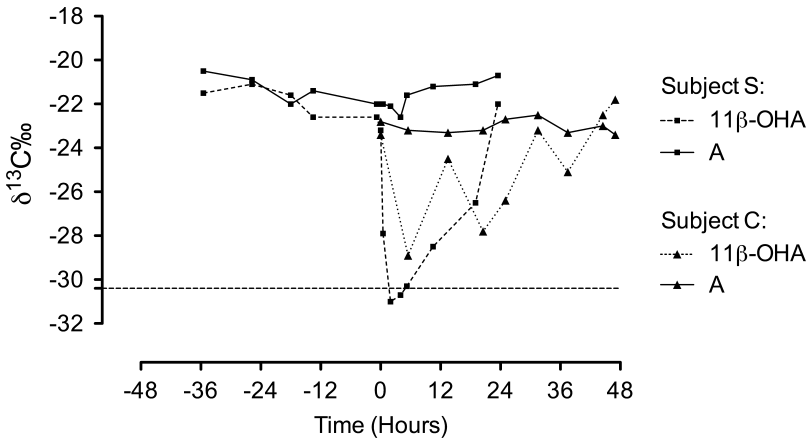

Figure 5. δ^{13}C excretion profiles of 11β-OHA and A after 11-OXO™ administration. Dashed line represents the δ^{13}C of the administered adrenosterone ($\delta^{13}C = -30.4 \pm 0.5$‰).

Reference population

The distribution of adrenosterone metabolite concentrations obtained from GC-MS steroid profiling is displayed in Figure 7. The median concentrations observed for 11β-OHA and 11β-OHEt were 320 and 141 ng/mL respectively. The maximum concentrations were 1492 (11β-OHA) and 1049 ng/mL (11β-OHEt). The mean 11β-OHA/11β-OHEt ratio determined from this data (3.6) was in good agreement with reported literature.[35,68] The median 11β-OHA/11β-OHEt ratio was 2.0, with a maximum observed value of 20.

The mean δ^{13}C values determined by GC-C-IRMS were −21.2‰ and −21.6‰ for 11β-OHA and A respectively (Figure 8). Both populations were normally distributed (D'Agostino and Pearson normality test, $p > 0.05$) and the minimum values observed were −22.8‰ (11β-OHA) and −23.7‰ (A). From these data $\Delta\delta^{13}$C values for 11β-OHA – A were constructed. The mean $\Delta\delta^{13}$C obtained (0.4‰) was in very good agreement with other published research on 11β-OHA – A.[56,63] A minimum $\Delta\delta^{13}$C of −1.5‰ and a maximum of 2.9‰were recorded.

Discussion

Development of anti-doping criteria

Analysis of the reference population GC-MS and GC-C-IRMS data highlights the changes observed during adrenosterone

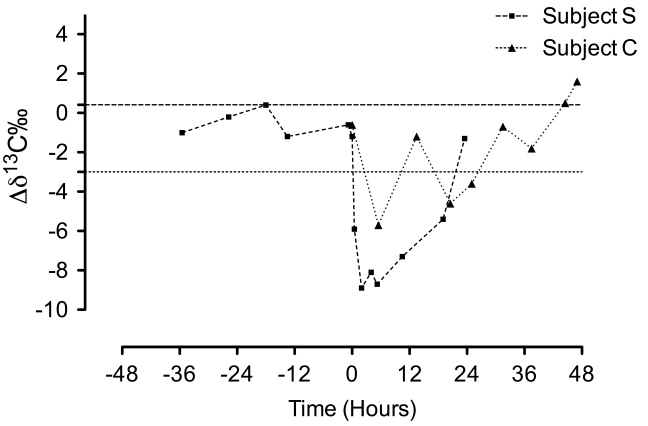

Figure 6. $\Delta\delta^{13}C$ excretion profiles of 11β-OHA – A after 11-OXO™ administration. Dashed lines represent the mean $\Delta\delta^{13}C$ of population profiling (mean $\Delta\delta^{13}C = 0.4$, $n = 85$) and a nominal (–) 3‰ cut-off.

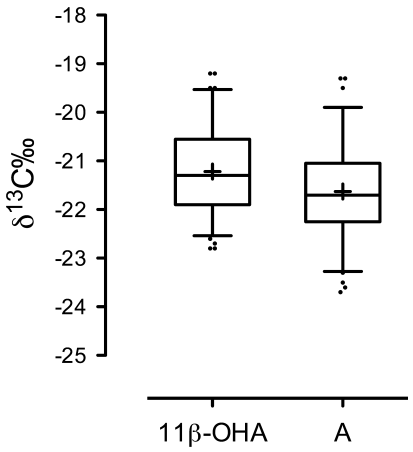

Figure 8. Boxplot of total reference population profiling of $\delta^{13}C$ for 11β-OHA and A ($n = 85$). Whiskers are 5–95 percentiles, Box is 25–75 percentiles, line is median, + is mean and dots are outliers.

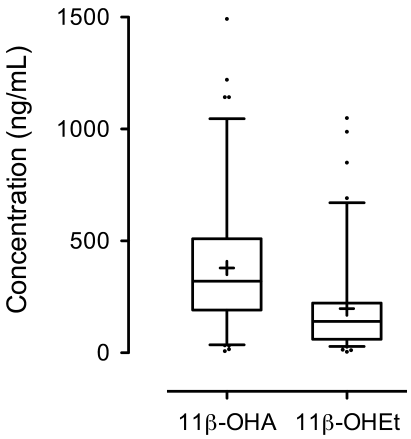

Figure 7. Boxplot of total reference population GC-MS steroid profiling for 11β-OHA and 11β-OHEt concentration (ng/mL, specific gravity adjusted to 1.020, $n = 85$). Whiskers are 5–95 percentiles, Box is 25–75 percentiles, Line is median, + is mean and dots are outliers.

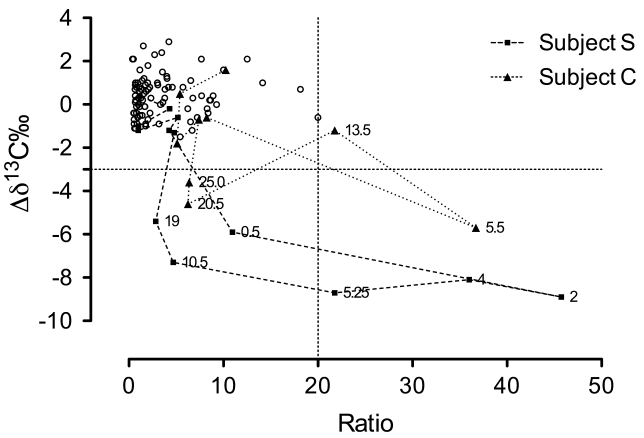

Figure 9. 2D plot of 11β-OHA/11β-OHEt ratio vs $\Delta\delta^{13}C$ of 11β-OHA – A for the reference population (open circles, $n = 85$) and selected 11-OXO™ administration study samples (Subject S and Subject C). The time after administration (hours) are annotated for each subject. Dashed lines represent a proposed 11β-OHA/11β-OHEt ratio threshold of 20 and a nominal $\Delta\delta^{13}C$ cut-off of (–) 3‰.

administration and allows anti-doping criteria to be proposed. The maximum 11β-OHA concentration determined after administration (155 µg/mL, i.e. 155 000 ng/mL, Figure 2) was approximately one hundred times greater than the maximum from the reference population (1492 ng/mL, Figure 7). Similarly, the elevated 11β-OHA/11β-OHEt ratio found after administration (46.7 and 36.7 for Subject S and C respectively) was significantly larger than the mean reference population value of 3.6. Due to the different major contributors to the 11-oxy-C_{19} metabolites, the 11β-OHA/11β-OHEt ratio can be considered as being indicative of the secretion and metabolism of 11β-hydroxyandrostenedione relative to the endogenous corticosteroids (Figure 1).[35] As these adrenal steroids can fluctuate considerably it may be expected that this ratio may vary much more than other steroid metabolite ratios, such as the testosterone to epitestosterone ratio (T/E).[43,69–71] Despite this, 11β-OHA/11β-OHEt ratios may prove to be very useful in addition to concentration data for screening for adrenosterone ingestion within long-term administration. With reference to the athlete population described above, previously established reference ranges,[68] and current WADA guidelines,[58] the following GC-MS steroid profiling screening criteria are proposed:

- 11β-OHA greater than 10 000 ng/mL (specific gravity adjusted to 1.020) *or*
- 11β-OHA/11β-OHEt ratio greater than 20.

Doping control urine samples flagged as suspicious by these criteria would be subjected to routine GC-C-IRMS confirmation procedures. Comparison of the various $\delta^{13}C$ and $\Delta\delta^{13}C$ values of appropriate 'ERC' (11β-OHA) and 'TC' (A) determined to relevant reference population statistics and appropriate WADA criteria[58] should ensure the identification of an adrenosterone doping violation. Figure 9 illustrates the interaction between the proposed GC-MS screening criteria and current WADA GC-C-IRMS confirmation criteria for both adrenosterone administration studies with relation to the reference population. It is clear that improved GC-MS screening would identify more samples that would be able to be confirmed by GC-C-IRMS. The identification of the adrenosterone metabolites 11-oxo-diol and compounds **Ia** and **IIa** introduces the possibility of incorporating specific screening markers into GC-MS steroid profiling procedures.[52] Indeed, markers for the prohormones androstenedione[4,72] and

dehydroepiandrosterone[50,51] have already been successfully introduced. These markers may significantly improve the detection period for identifying suspicious samples when used in conjunction with traditional steroid profiling.

Issues to consider for doping control

As adrenosterone is similar in chemical structure and biological effects to other anabolic androgenic steroids that are listed on the WADA 2009 Prohibited List, it should be considered for prohibition for use in sport.[19] The 11β-HSD1 enzyme supposedly targeted by adrenosterone supplementation also plays an important role in metabolic diseases such as diabetes and obesity.[30] Consequently, specific 11β-HSD1 inhibitors have become targets for pharmaceutical development.[73] Therefore the progress in the development of non-steroidal 11β-HSD1 inhibitors as therapeutic drugs should be monitored for their potential as doping agents.

It has been demonstrated that ERCs such as the 11-oxy-C_{19} metabolites can be used for the GC-C-IRMS confirmation of adrenosterone as well as endogenous corticosteroid administration.[59,60] Similarly it has be shown that pregnenolone administration leads to significant $\delta^{13}C$ changes in another often used ERC, pregnanediol.[74] These findings emphasize the need for GC-C-IRMS methods utilized for the confirmation of endogenous anabolic androgenic steroid administration to have access to multiple ERC from different metabolic pathways to alleviate the possibility of confounding interferences due to the co-administration of other endogenous steroids.

Conclusion

We reported the detection and confirmation of ten metabolites of adrenosterone by GC-MS following the administration of an 11-OXO™ dietary supplement (Table 1). Significant changes in the urinary steroid profile were observed within 24 hours of administration. Routine GC-C-IRMS confirmation methods were utilized to establish the exogenous origin of the major adrenosterone metabolite present in post-administration study urines. Analysis of a reference population data set enabled anti-doping screening criteria to be proposed. The identification of novel adrenosterone metabolites introduces the possibility of incorporating specific markers into GC-MS steroid profiling for improved anti-doping screening criteria.

Acknowledgements

The authors would like to thank Prof. Mario Thevis and *Drug Testing and Analysis* for the invitation to prepare this manuscript for the 27th Cologne Workshop on Doping Analysis special issue. The Henry Bertie and Florence Mabel Gritton Postgraduate Research Scholarship from the University of Sydney, the German Federal Ministry of the Interior, and the Manfred-Donike-Institute for Doping Analysis, Cologne are acknowledged for financial support. Additionally, the support and assistance of all the staff members of the Australian Sports Drug Testing Laboratory is noted, and especially with regards to this work, Jan Tjoa, Susan Soo and Ronald Huynh.

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