Ahrens BD, Butch AW

Steroid profiles and isotope-ratio mass spectrometry analysis of various metabolites and endogenous reference compounds. The UCLA experience.

UCLA Olympic Analytical Laboratory, Department of Pathology & Laboratory Medicine, Geffen School of Medicine at UCLA, Los Angeles, CA, U.S.A.

Introduction

Current practice in our laboratory is to conduct isotope-ratio mass spectrometry (IRMS) analysis of androsterone (Andro), etiocholanolone (Etio) and 5β-pregnane-3α,20α-diol (Pdiol) on samples having atypical steroid profiles[1] as measured by gas chromatography/mass spectrometry (GC/MS) and when requested by a client (manifest requests). During the past 18 months 2,904 IRMS tests were performed by our laboratory. IRMS testing was conducted on 817 urine samples (28%) due to atypical steroid profiles. The remaining samples were manifest requests for IRMS analysis. Given our test volumes and, as previously observed by others, because of the need for a second liquid chromatography clean-up to achieve adequately clean extracts for some analytes,[2] we do not routinely perform an analysis of epitestosterone (E), dehydroepiandrosterone (DHEA), 5β-androstane-3α,17β-diol (Bdiol) and 5α-androstane-3α,17β-diol (Adiol) or alternative endogenous reference compounds (ERCs) including 11-hydroxyandrosterone (OHA), 11-ketoetiocholanolone (K) and 5α-androst-16-en-3α-ol (16EN) with every IRMS request. In particular for samples where the steroid profile is in not any way atypical, criteria to select samples for further IRMS workup based upon results for Andro and Etio may be useful.

Materials and Methods

Samples. 2904 athlete urines were collected by a NADO either in- or out-of-competition during an 18 month period beginning in the middle of 2009 and were analyzed by IRMS. Quality controls for IRMS were prepared by pooling urine collections from a single individual known not to be taking prohibited substances and by fortifying the urine with target steroids

to prepare two pools with δ-values consistent with endogenous origin and with steroid administration, respectively.

IRMS analysis. Two quality control urines handled in the same manner as unknown urines were included with each batch of 1 to 18 unknown urines. After enzymatic hydrolysis and centrifugation, the supernatants were applied to Bond-Elut solid-phase extraction (SPE) columns (Varian, USA) with a 500 mg bed size preconditioned with methanol and water. After washing with water steroids were eluted with methanol/ethyl acetate (30:70, v/v) and the solvent was evaporated. After reconstitution in 100 μL methanol and removal of insoluble material on Costar spin-x filters (Sigma, USA), the extracts were transferred to chromatographic vials and reconstituted in 100 μL of acetonitrile/water (50:50, v/v) containing estradiol diacetate. All cleanups were performed on an 1100-series HPLC with autoinjector, diode-array detector and preparative-scale fraction collector (Agilent, USA). Prior to injection of samples on the HPLC, triplicate injections of a standard containing the steroids of interest were required to have retention times for each steroid not varying more than 0.05 minutes. Sample extract volumes of 90 μL were autoinjected onto an Onyx Monolithic 100 x 4.6 mm C18 column (Phenomenex, USA) at a flow rate of 3 mL/minute running a gradient initially 30% acetonitrile (B) in water (A), ramped to 50% B over 6 minutes, ramped to 95% B over 0.5 minutes and held at 95% B for 2 minutes and then decreased to 30% B over 0.5 minutes and held for 3 minutes to allow equilibration prior to the next injection. Six time-based fractions were collected; specifically OHA and K in fraction 1, T in fraction 2, DHEA, Epit , Adiol and Bdiol in fraction 3, Andro and Etio in fraction 4, Pdiol in fraction 5 and 16EN in fraction 6 (Figure 1). Fractions 1, 2 and 6 were reconstituted in 25 μL cyclohexane containing 5α-androstan-3β-ol at 40 μg/mL (RS). Fraction 4 was reconstituted in 100 to 300 μL RS depending upon known concentrations of Andro and Etio. The steroids in fractions 3 and 5 were derivatized to their corresponding acetates in 50 μL pyridine and 50 μL acetic anhydride at 60 ºC. Fraction 5 was reconstituted in 50 μL RS.

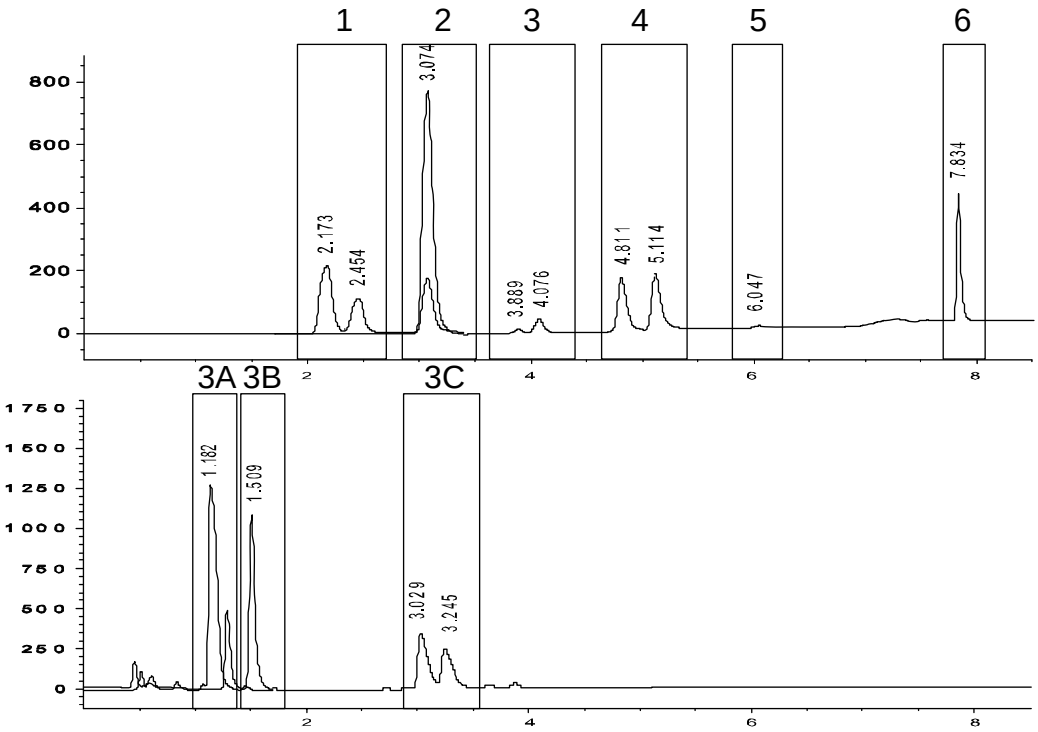

Figure 1. HPLC fraction collection intervals. First cleanup: OHA and K (1), T (2), Epit, DHEA, Adiol and Bdiol (3), Andro and Etio (4), Pdiol (5) and 16EN (6). Second cleanup: Epit acetate (3A), DHEA acetate (3B) and Adiol diacetate and Bdiol diacetate (3C).

Fraction 3 was reconstituted in 100 μL acetonitrile/water (70:30 v,v) and subjected to a further HPLC cleanup by autoinjection using the same flow rate, injection volume, and column as the first cleanup. The gradient, initially 70% acetonitrile (B) in water (A), was ramped to 95% B over 6 minutes and held at 95% B for 2.5 minutes and then decreased to 70% B over 1.5 minutes and held for 3 minutes to allow equilibration prior to the next injection. Three time-based fractions were collected; specifically Epit in fraction 3A, DHEA in fraction 3B and Adiol and Bdiol in fraction 3C (Figure 1). Fractions 3A, 3B and 3C were reconstituted in 25 μL RS.

All IRMS analyses were performed on a Trace Ultra GC (Thermo Fisher, USA) coupled to both a DSQ-II single quadrupole MS (Thermo Fisher, USA) and a GC Combustion III (Thermo Fisher, USA) interfaced to a Delta V Plus IRMS (Thermo Fisher, USA). A portion of the reconstituted extracts ranging from 0.3 to 3 μL were injected at 280 ºC with a splitless time of 0.6 minutes onto a HP-50+ column having a 30 m length, 0.25 mm internal diameter and 0.15 μm film thickness with an approximate flow rate of 1.5 mL/minute. The MS was operated in full-scan mode monitoring m/z 50-450. The combustion furnace was operated at 940 ºC and received a low flow of oxygen for 30 seconds after each analysis. Traceability for

all δ-values was achieved through CU/USADA 34-1[3] used to calibrate a cylinder of CO_2 (Praxair, USA). Pulses of CO_2 were introduced directly into the IRMS source prior to and following the elution region of the steroids of interest. In-house standards containing the steroids of interest with δ-values characterized against CU/USADA 34-1 were injected prior to and after analysis of unknowns and associated quality control urines.

Results

The steroid profiles for 785 samples for which IRMS analysis was requested by the client were normal in that they did not exceed any specified thresholds for endogenous steroids. For these samples the IRMS results for Δδ(Pdiol-Andro) and Δδ(Pdiol-Etio) were distributed around means of -0.16 (0.69) and 1.09 (0.67), respectively (Figure 2). Both distributions exhibit slight negative skewness and kurtosis excess. For these samples the 95th percentiles for Δδ(Pdiol-Andro) and Δδ(Pdiol-Etio) were determined to be 1.0 and 2.2, respectively (Figure 2).

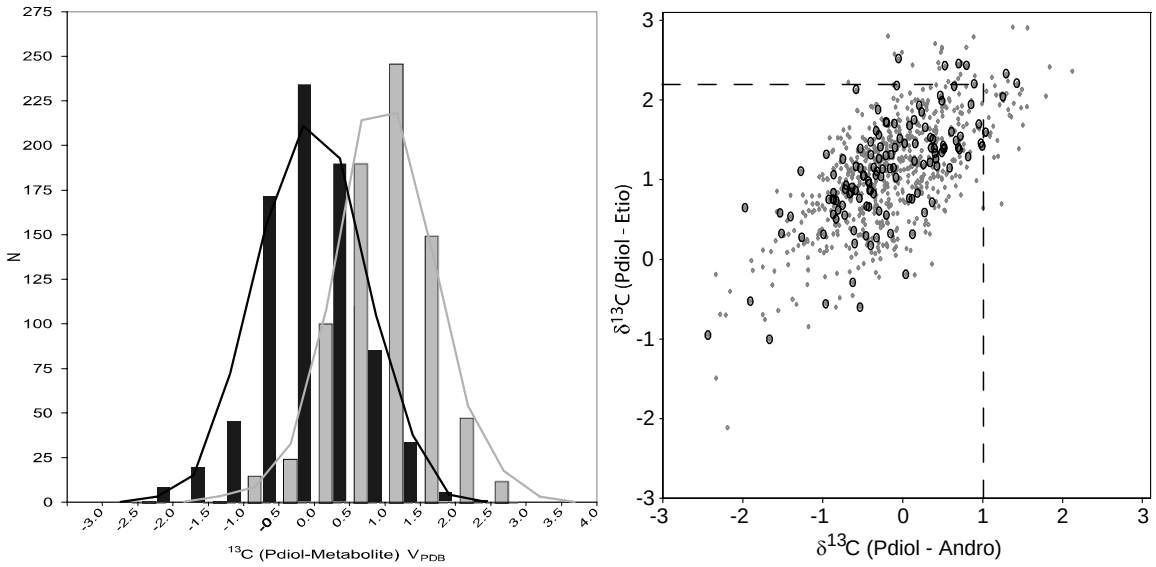

Figure 2. Left Panel: Distribution of Δδ-values for Andro (dark) and Etio (light) for 785 samples with normal steroid profiles. Lines represent fitted distributions. Right Panel: Correlated Δδ-values for Andro and Etio. Circled observations and dashed lines indicate steroid profile outliers and 95th percentiles, respectively.

Subsequently, all samples for which these limits were exceeded had further IRMS analysis of Adiol and Bdiol. During the ensuing 10 months, 22 samples exceeded the threshold for Etio and 6 of these samples also exceeded the threshold for Andro. Although none of the 22 samples were adverse based on Andro or Etio, 1 of the samples was adverse based on the

diols analysis with Δδ(Pdiol-Bdiol) and Δδ(Pdiol-Adiol) of 6.4 and 6.0, respectively. Overall, IRMS testing resulted in 37 (1.3%) adverse analytical findings. An elevated T/E or elevated DHEA in the absence of other atypical observations accounted for 15 and 7 of the adverse analytical findings, respectively. Two samples had both an elevated T/E ratio and DHEA concentration. In each of the 8 adverse cases where Andro and/or Etio were atypical, either the T/E ratio or DHEA concentration was also atypical. The remaining 5 cases were manifest requests. Four of the adverse findings illustrate the variability encountered when evaluating combinations of endogenous reference compounds and metabolites (Table 1).

Table 1. Illustrative cases: T/E ratio, steroid concentrations and IRMS results.

	CASE	1	2	3	4
	T/E ratio	9.2	8.2	1.2	1.2
DHEA	ng/mL	83	116	119	221
T	ng/mL	128	14	55	83
	Δδ vs. Pdiol (‰)	10.6	9.4	2.6	
Andro	ng/mL	5417	4287	4043	7646
	Δδ vs. Pdiol (‰)	6.1	10.0	4.7	11.2
Etio	ng/mL	7066	3888	4371	4422
	Δδ vs. Pdiol (‰)	7.9	9.3	5.5	8.9
Adiol	ng/mL	145	55	55	186
	Δδ vs. Pdiol (‰)	11.4	10.1	2.9	7.4
Bdiol	ng/mL	510	158	520	274
	Δδ vs. Pdiol (‰)	9.9	7.2	3.4	4.2
Pdiol	$\delta^{13}C$ (‰)	-18.9	-20.1	-22.8	-27.3
OHA	$\delta^{13}C$ (‰)	-18.1	-19.4	-17.8	-19.1
16EN	$\delta^{13}C$ (‰)	-18.6	-19.5	*	*
K	$\delta^{13}C$ (‰)	-18.2	-27.2	-22.7	*

* Not measured due to low concentration of the ERC.

It is expected that unless a precursor of an ERC has been administered the δ-values for ERCs will be similar as is the case for case 1. However, in the case of athlete 2, K is apparently significantly ^{13}C-depleted relative to Pdiol, OHA and 16EN. Additionally for case 2, Andro, Etio, Adiol and Bdiol are similarly ^{13}C-depleted while for case 1, Adiol and Bdiol are more depleted than Andro and Etio. The T/E for cases 1 and 2 were 9.2 and 8.2, respectively.

Cases 3 and 4 both exhibited DHEA concentrations greater than 100 ng/mL and in both cases Andro and Etio were more ^{13}C-depleted than either Adiol or Bdiol. Case 4 also exhibits an unusually ^{13}C-depleted Pdiol and in this case the athlete later admitted to co-administration of DHEA and pregnenolone. The observed result for Pdiol in this case has since been confirmed by others in a controlled administration study of pregnenolone.[4]

Summary

Although the majority of IRMS-based adverse findings reported by our group are for samples that exhibit atypical steroid profiles, it is not surprising that cases where IRMS results indicate administration of a steroid accompany a normal steroid profile. It is possible that these cases would be atypical from the perspective of longitudinal analysis but this information is quite often not available at the time of IRMS analysis. Being aware that the profile from a single collection is not always sensitive to steroid administration, testing authorities may continue looking to IRMS analysis. Should this result in a continued increase in demand for IRMS testing, results from an easily accessible Andro and Etio analysis, coupled with criteria for additional testing may be prove useful in focusing the efforts of anti-doping laboratories.

Acknowledgement

CU/USADA 34-1 was provided by the United States Anti-doping Agency

References

[1] World Anti-Doping Agency. Reporting and Evaluation Guidance for Testosterone, Epitestosterone, T/E Ratio and Other Endogenous Steroids. Technical Document, Montreal (2004) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2004EAAS_EN.pdf (access date 8.01.2011)

[2] Piper T, Mareck U, Geyer H, Flenker U, Thevis M, Platen P, Schänzer W. (2008) Determination of $^{13}C/^{12}C$ ratios of endogenous urinary steroids: method validation, reference population and application to doping control purposes. *Rapid Commun. Mass Spectrom.* **22**, 2161-2175.

[3] Zhang Y, Tobias H, Brenna T. (2009) Steroid isotopic standards for gas chromatography-combustion isotope ratio mass spectrometry. *Steroids* **74**, 369-378.

[4] Piper T, Schlug C, Mareck U, Schänzer W. (2011) Investigations on changes in $^{13}C/^{12}C$ ratios of endogenous urinary steroids after pregnenolone administration. Presented at the Manfred Donike Workshop, 29th Workshop on Dope Analysis, Köln Germany.