Ahrens B, Butch A

Preliminary data from steroid administration studies for establishing local IRMS Δδ^{13}C threshold values to enhance detection of endogenous steroid use.

Pathology and Laboratory Medicine, UCLA, Los Angeles, United States

Abstract

Carbon isotope ratio mass spectrometry (IRMS) testing is performed to determine if an atypical steroid profile or atypical testosterone/epitestosterone (T/E) ratio is the result of administration of an endogenous steroid. Administration studies with three steroid formulations were performed to determine if currently applied Δδ^{13}C thresholds for various compounds are optimized for sensitivity. Twelve subjects (4 per group) with high mode urinary T/E ratios received either 20 mg of transdermal testosterone (T patch), 120 mg testosterone undecanoate (oral T) or 100 mg of oral dehydroepiandrosterone (DHEA) on days 2, 3 and 4 of a 6 day study. Urine was collected in 4-hour intervals throughout the day (3 collections) and in a 12-hour interval over night. Urinary steroid profiles and δ^{13}C values of androsterone (Andro), etiocholanolone (Etio), 5α-androstanediol (Adiol), 5β-androstanediol (Bdiol) and pregnanediol were measured by gas chromatography-mass spectrometry and IRMS, respectively. 5α-androstanediol, 5β-androstanediol, and 5β-androstanediol/etiocholanolone Δδ^{13}C values were found to be the most sensitive markers for T patch, oral T and DHEA use, respectively. After oral T and DHEA administration the Δδ^{13}C values of Adiol, Bdiol, T and DHEA never exceeded 3‰ when Andro and/or Etio Δδ^{13}C values were below the established thresholds for triggering androstanediol testing. Following T patch administration there were 2 time points when Adiol exceeded 3‰ without the Andro and Etio thresholds being exceeded. However, Adiol values at these 2 time points never exceeded 3‰ plus the laboratory measurement uncertainty and would not have been considered adverse.

Introduction

IRMS testing, when employed due to an atypical steroid profile or upon request by a testing authority, utilizes Δδ^{13}C thresholds, which when exceeded confirms that testosterone (T) or a T precursor has been administered. Our laboratory routinely determines δ^{13}C values of androsterone (Andro), etiocholanolone (Etio) and the endogenous reference compound (ERC) 5β-pregnane-3α,20α-diol (Pdiol), and in cases where the results are not adverse but the Δδ^{13}C values exceed established thresholds, proceed to measure the δ^{13}C values of 5α-androstane-3α,17β-diol (Adiol) and 5β-androstane-3α,17β-diol (Bdiol) [1]. A similar strategy has also been used to establish a threshold for Pdiol such that an alternative ERC could be used to establish the true endogenous reference value. Since 5α-androstane-3α,17β-diol (Adiol) has been shown to be a more sensitive indicator of testosterone (T) gel use [2] steroid administrations with different formulations were undertaken to determine if our local Δδ^{13}C Andro (1.0‰) and Etio (2.2‰) thresholds were appropriate for triggering androstanediol (Diols) analysis for optimal detection of endogenous steroid use. The Δδ^{13}C values for Andro, Etio, Diols, T, and DHEA were evaluated to determine if any of the administration protocols would lead to an adverse Adiol, Bdiol, T or DHEA Δδ^{13}C value when Andro or Etio failed to exceed the local thresholds that would trigger additional IRMS testing.

Experimental

Male subjects ranging from 18 to 30 years of age in good health who had previously provided 3 untimed urine collections at least one week apart with a T/E ratio >0.3 were randomly divided into three groups (4 subjects per group) that received either 100 mg DHEA orally (AST Sports Science, Golden, CO, USA), 120 mg of testosterone undecanoate orally (75.9 mg of

 ISBN 978-3-86884-039-1

testosterone, Testocap®, Schering-Plough, Whitehouse Station, NJ, USA), or 20 mg of testosterone transdermally by applying adhesive patches to the skin (Androderm®, Watson Pharma, Parsippany, NJ, USA). Steroid administration took place at the start of the first 4-hour collection on days 2, 3 and 4. On each day of the study all urine was collected during three 4-hour intervals and one 12-hour overnight interval until day 6 when only two 4-hour urines were collected. The administration studies were approved by the Office of the Human Research Protection Program at UCLA.

Urinary steroid concentrations were determined using our standard gas chromatography-mass spectrometer (GC-MS) procedure for exogenous and endogenous steroids with the addition of d_3-epitestosterone, d_5-DHEA, d_3-Adiol, d_3-Bdiol, d_4-Andro glucuronide and d_5-Etio to the internal standard cocktail for measuring steroid concentrations [3].

Sample preparation for IRMS included hydrolysis with β-glucuronidase, solid-phase extraction with Bond Elut C18 LRC cartridges (Agilent, Santa Clara, CA, USA), and an HPLC clean-up (1100 series, Agilent) as described [1]. Collected fractions containing Adiol, Bdiol, DHEA and Pdiol were derivatized to their corresponding acetates. The fraction containing Adiol, Bdiol, and DHEA underwent a second HPLC clean-up step. A Trace Ultra GC coupled to a DSQ-II single quadrupole MS and a GC Combustion III furnace that was interfaced to a Delta V Plus IRMS (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine steroid $\Delta\delta^{13}C$ values as previously described [1].

Content analysis of DHEA and T capsules was performed by extracting the contents of selected capsules with 10 mL of methanol (HPLC grade, Fisher Scientific, Pittsburgh, PA, USA). The extracts were loaded onto a HPLC system with auto injector and diode-array detector (1100 series, Agilent) and analyzed by monitoring the signal of a standard curve and the capsule extracts at 215 nm. The central portion of a T patch, excised from the outer adhesive region, was suspended overnight in 25 mL of 2-propanol at ambient temperature for T extraction. Prior to GC-MS and IRMS analysis, a portion of the Oral T extract was hydrolyzed using trimethylchlorosilane to cleave the ester [4]. GC-MS and IRMS analysis of the steroid preparations was then performed to determine the concentration and $\Delta\delta^{13}C$ value of each steroid preparation.

Changes in steroid concentrations and their ratios, $\Delta\delta^{13}C$ values and $\Delta\delta^{13}C$ values with respect to Pdiol for each collection interval were averaged for the 4 subjects that completed the administration protocol for DHEA, oral T and T patch. Urinary steroid concentrations were normalized to a specific gravity of 1.020 before calculation of averages.

Results and Discussion

DHEA and T capsules were found to contain an average of 92.0 (SD of 8.0; n=5) and 38.0 (SD of 0.2; n=4) mg of DHEA and testosterone undecanoate, respectively [4]. No off label steroids were identified in any of the steroid preparations. The $\delta^{13}C$ values for DHEA, hydrolyzed T from testosterone undecanoate and T in the T patch (mean ±SD; n=3) were -31.0 ±0.1, -30.2 ±0.1 and -28.5 ±0.1‰, respectively.

Baseline $\delta^{13}C$ values for each participant prior to administration of the steroid are provided in Table 1.

Analyte	DHEA[a]	Oral T[b]	T Patch[c]
Andro	-18.7, -18.3, -18.3, -17.9	-18.3, -19.9, -20.0, -20.2	-18.2, -18.0, -20.0, -19.2
Etio	-19.9, -18.6, -19.4, -19.4	-19.9, -21.1, -21.9, -22.0	-19.4, -18.1, -20.9, -20.2
Adiol	-20.4, -20.2, -21.1, -19.6	-20.0, -21.6, -21.9, -20.9	-20.1, -20.6, -22.6, -21.8
Bdiol	-19.9, -19.6, -19.7, -19.9	-19.7, -21.1, -22.3, -20.3	-18.8, -18.6, -20.7, -20.0
Pdiol	-19.3, -18.2, -19.0, -19.1	-19.0, -21.0, -20.9, -19.5	-18.9, -18.7, -20.6, -19.9
T	-19.7, -19.3, -18.2, -20.3	-19.2, -20.3, -21.1, -19.2	-18.4, -16.7, -20.3, -19.6
DHEA	-19.0, -17.0, -17.4, -17.1		

[a,b,c] Values for each of four subjects obtained prior to steroid administration.

Table 1: Baseline urinary $\Delta\delta^{13}C$ values

 ISBN 978-3-86884-039-1

Within the first 4 hours following oral DHEA administration, $\Delta\delta^{13}C$ values of Andro, Etio, Adiol, Bdiol, DHEA and T all exceeded 3 ‰. At this time point Andro, Etio and DHEA $\Delta\alpha^{13}C$ values were the largest at 7.6, 8.4 and 9.0 ‰, respectively (Figure 1). Maximum $\Delta\alpha^{13}C$ values for Adiol, Bdiol and T were reached between 4 and 12 hours following DHEA administration. A similar pattern was observed following administration of DHEA on days 2 and 3. With few exceptions the $\Delta\alpha^{13}C$ values for all of the monitored steroids declined during the 12-hour overnight collections but remained above baseline values. Throughout the time course (8 hours post-administration of the first dose), Etio and Bdiol exhibited the largest $\Delta\alpha^{13}C$ values and they remained above the threshold value of 3 ‰ for 56 hours after the last administered dose. Concentrations of Andro, Etio, Adiol, Bdiol, DHEA and T all increased following each dose of DHEA, with the largest increases in the 5β-metabolites Etio and Bdiol. Decreases in the ratios of Andro/Etio and Adiol/Bdiol concentrations relative to baseline were observed 12 hours after the first dose and continued until 48 hours after the last dose. Although the ratio of DHEA/epitestosterone increased almost 10-fold and remained elevated for 12 hours following each dose, the ratio dropped near baseline 24 hours after dosing. Average baseline T/E ratios were slightly >1 and did not exceed 2.5 at any time.

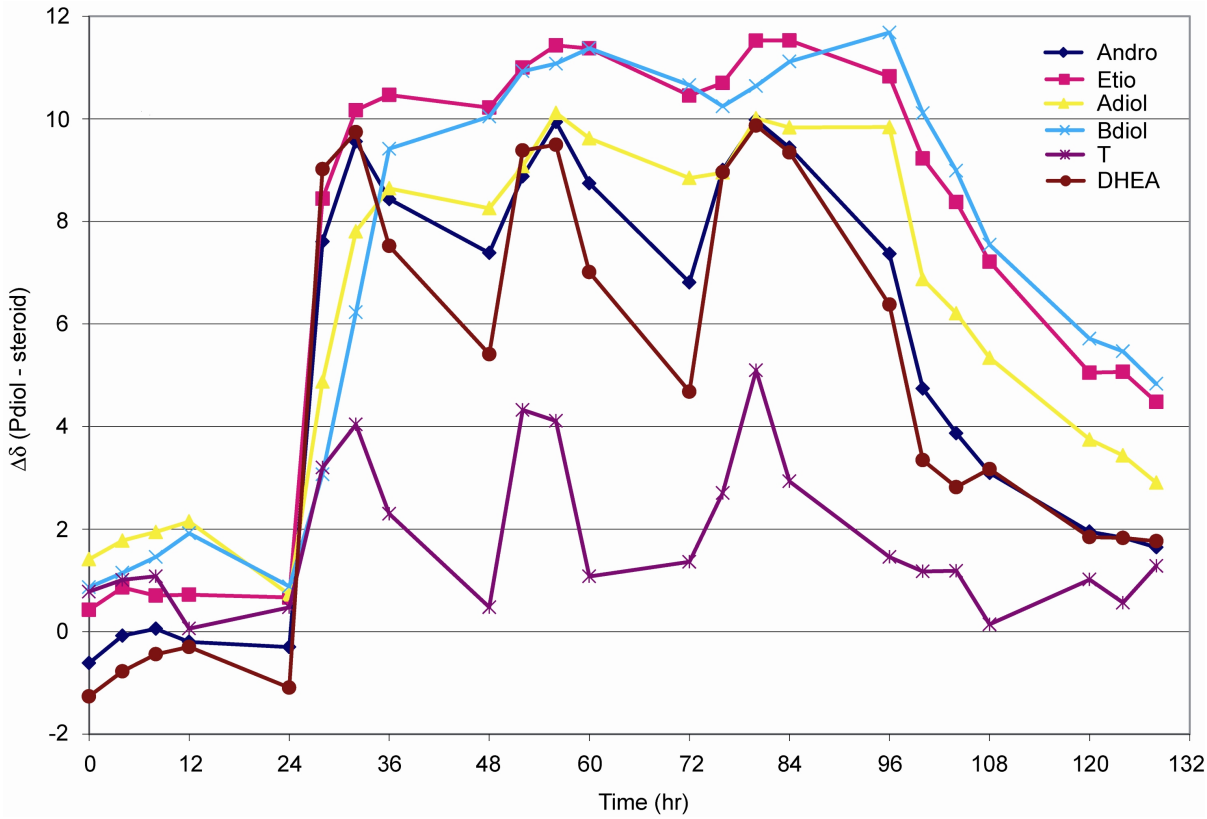

Figure 1. Steroid $\Delta\delta^{13}C$ values at various time periods following oral administration of 100 mg of DHEA. DHEA was administrated at the 24, 48 and 72 hour time points. Urine was collected in 4-hour time intervals during the day and in a single 12-hour over night collection. Each data point is the average $\Delta\delta^{13}C$ value from 4 subjects.

Following oral T administration, $\Delta\alpha^{13}C$ values of Andro, Etio, Adiol, Bdiol and T exceeded 3 ‰ within 4 hours after administration, peaked at 8 hours, and were declining at 12 hours. Although $\Delta\alpha^{13}C$ values for Andro and T fell below 3 ‰ during the 12-hour overnight collection, Etio, Adiol and Bdiol $\Delta\alpha^{13}C$ values remained elevated. This pattern continued following the second and third dose of oral T, however, the $\Delta\alpha^{13}C$ value for Bdiol alone was slightly above the 3 ‰ threshold 36 hours after the last dose. Adiol and Bdiol concentrations and the Adiol/Bdiol ratio were similar after oral T administration to those observed for DHEA. Andro and Etio concentrations increased in response to oral T but the increases were evident for a shorter period of time compared to those observed for DHEA. Only a modest effect on the Andro/Etio ratio was observed in response to oral T. T/E ratios increased dramatically with average ratios exceeding 30 in the first 4 hours following each dose.

ISBN 978-3-86884-039-1

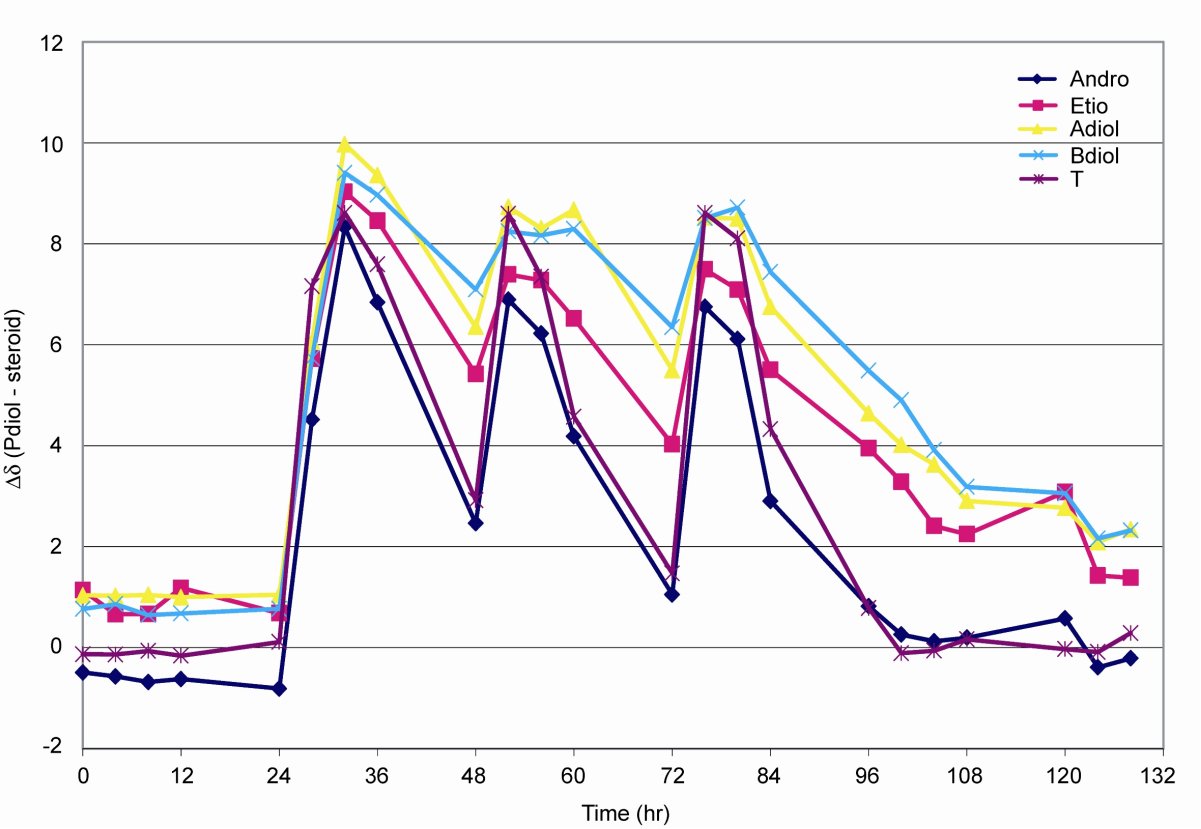

Figure 2. Steroid $\Delta\delta^{13}C$ values at various time periods following oral administration of 120 mg of testosterone undecanoate. Testosterone undecanoate was administrated at the 24, 48 and 72 hour time points. Urine was collected in 4-hour time intervals during the day and in a single 12-hour over night collection. Each data point is the average $\Delta\delta^{13}C$ value from 4 subjects.testosterone undecanoate. Testosterone undecanoate was administrated at the 24, 48 and 72 hour time points. Urine was collected in 4-hour time intervals during the day and in a single 12-hour over night collection. Each data point is the average Dd value from 4 subjects.

As expected following T patch administration, $\Delta\alpha^{13}C$ values of Andro, Etio, Adiol, Bdiol and T increased more slowly compared to administration of the two oral drugs. The $\Delta\alpha^{13}C$ values did not peak until 12 hours after application of the patches and unlike the response to DHEA and oral T, the $\Delta\alpha^{13}C$ values for T remained above 3‰ from 4 hours after the first dose until 28 hours following the last dose. Adiol showed the greatest response to T patch administration being above the $\Delta\delta^{13}C$ value threshold of 3‰ at all time points up to 48 hours following the last dose. Andro and Etio showed smaller increases in $\Delta\delta^{13}$ C values, however, it was notable that baseline $\Delta\delta^{13}C$ values for Adiol were nearly 2‰ greater than all the other analytes. Increases in Andro and Etio concentrations were not as evident as those observed for the oral steroids and never exceeded the threshold concentration of 10,000 ng/mL. Adiol and Bdiol concentrations increased 2-fold over baseline at several time points but the pattern was not consistent during drug administration. Adiol/Bdiol and Andro/Etio ratios remained mostly unchanged except after the second application of the T patches and a day after administration was discontinued. During these two time intervals the ratios sharply increased, which is the opposite of what was observed following DHEA and oral T administration. Eight hours after the first dose the T/E ratio was above 4 and stayed above this threshold until 24 hours after the last dose.

ISBN 978-3-86884-039-1

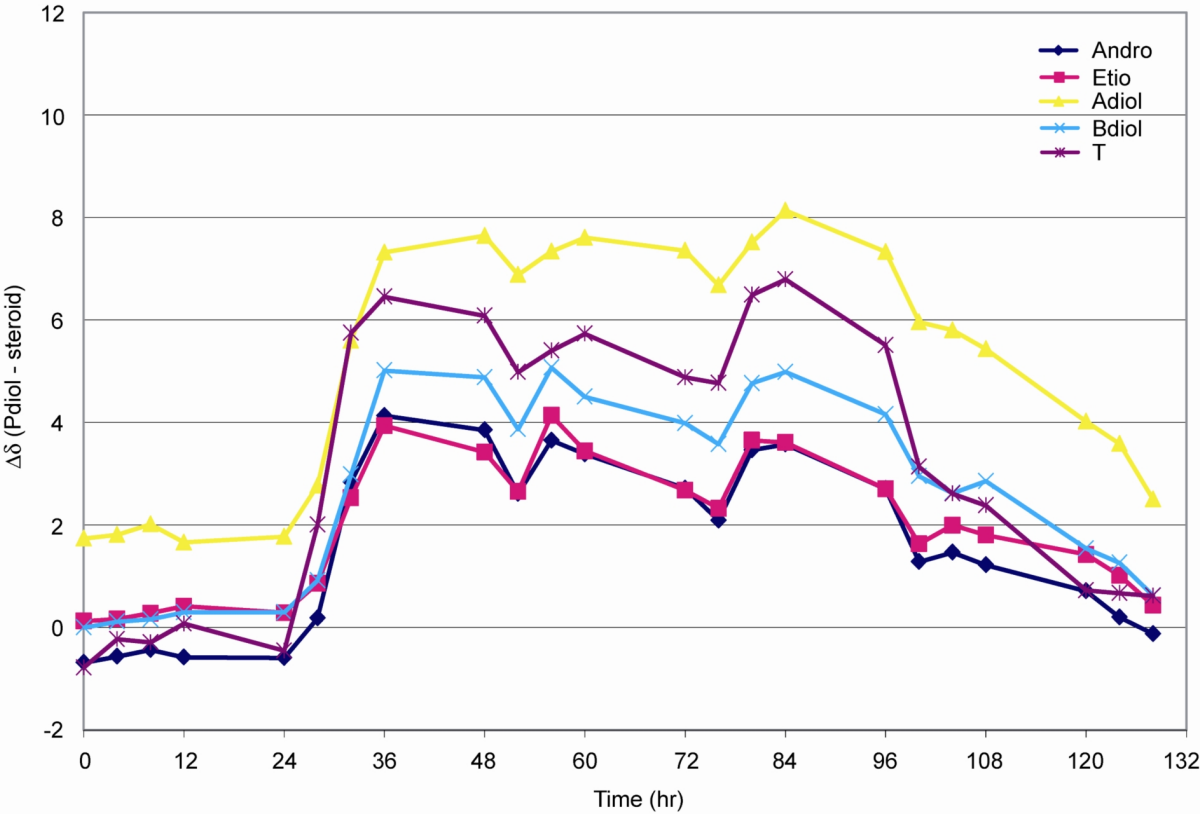

Figure 3. Steroid $\Delta\delta^{13}C$ values at various time periods following transdermal application of 20 mg of testosterone (T patch). T patches were applied to the skin at the 24, 48 and 72 hour time points. Urine was collected in 4-hour time intervals during the day and in a single 12-hour over night collection. Each data point is the average $\Delta\delta^{13}C$ value from 4 subjects.

Conclusions

After administration of oral DHEA or T, the $\Delta\delta^{13}C$ values for Adiol, Bdiol, DHEA and T never exceeded 3‰ when $\Delta\delta^{13}C$ values for Andro or Etio were below locally established thresholds that would trigger additional IRMS testing (Diols). Following application of transdermal T there were 2 time points when Adiol exceeded 3‰ without triggering Diols testing based on Andro and Etio thresholds. However, the Adiol $\Delta\delta^{13}C$ values at these time points failed to exceed 3‰ plus measurement uncertainty and would not have been considered adverse. Andro/Etio threshold values for triggering Diols testing will need to be re-evaluated, especially if threshold $\Delta\delta^{13}C$ values for Diols are lowered or if measurement uncertainty is not applied to the WADA threshold values.

References

[1] Ahrens, B. D. and Butch, A. W. (2013), Carbon isotope ratio mass spectrometry for detection of endogenous steroid use: A testing strategy. Drug Test Analysis, 5:534–540. doi:10.1002/dta.1447
[2] Piper, T., Mareck, U., Geyer, H., Flenker, U., Thevis, M., Platen, P. and 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. doi:10.1002/rcm.3601
[3] Breidbach, A. and Catlin, D. H. (2001), RSR13, a potential athletic performance enhancement agent: detection in urine by gas chromatography/mass spectrometry. Rapid Commun. Mass Spectrom., 15:2379–2382. doi:10.1002/rcm.523

[4] Cawley, A., Collins, M., Kazlauskas, R., Handelsman, D. J., Heywood, R., Longworth, M. and Arenas-Queralt, A. (2010), Stable isotope ratio profiling of testosterone preparations. Drug Test. Analysis, 2:557-567.

Acknowledgements

We are grateful to the Partnership for Clean Competition for funding this ongoing study and would like to acknowledge Fang Liu for her work in sample preparation and analysis.