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## **Co-administration of dietary supplements containing dehydroepiandrosterone and pregnenolone: Investigation of the impact on routine GC-C-IRMS doping control results.**

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### **Abstract**

Products containing dehydroepiandrosterone and pregnenolone are available on the dietary supplement market. Supposed benefits include increased mood and mental function, in addition to athletic performance enhancement. While combined use would not influence the efficacy of GC-MS steroid profiling, it will severely affect routine GC-C-IRMS methods that utilise pregnanediol as the endogenous reference compound. An open, non-randomized interventional study in healthy volunteers (4 male, 4 female) was conducted to obtain urine samples for steroid analysis. For all volunteers, three spot urine samples were collected at these time points: before administration; after 3 days of 100 mg DHEA/day only; and after an additional 3 days of 100 mg DHEA/day with 200 mg PREG/day (male) or 2.5 mg Letrozole/day (female).

As expected, IRMS results for all female post-administration samples were classified as positive according to WADA TD2004EAAS criteria (i.e.  $\Delta^{13}\text{C}_{\text{ERC-TC}} > 3 \text{‰}$ ). Three out of four male samples provided after the first administration of DHEA were also positive, however samples collected after co-administration were not due to the influence of the metabolism of pregnenolone on the ERC pregnanediol. Further steroid profiling and IRMS results are discussed in relation to the impact on routine doping control and some solutions are suggested.

### **Introduction**

Products containing both dehydroepiandrosterone (3 $\beta$ -hydroxyandrost-5-en-17-one, DHEA) and pregnenolone (3 $\beta$ -hydroxypregn-5-en-20-one, PREG) are increasingly available on the dietary supplement market. Supposed benefits for taking this combination include increased mental function (e.g. learning and memory) in addition to assumed athletic performance enhancement [1]. With regards to doping, the use of a combined DHEA and PREG product may be to promote the supposed "backdoor pathway" of DHT production, rather than via the conventional  $\Delta 4$  or  $\Delta 5$  pathways [2].

While the combined use of DHEA with PREG should not influence the efficacy of GC-MS steroid profiling, it will adversely affect routine GC-C-IRMS methods that utilise pregnanediol (5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol, PD) as the endogenous reference compound (ERC) [3-5].

### **Experimental**

An investigation was conducted on the impact of co-administration on routine anti-doping procedures used at ASDTL for the control of endogenous anabolic androgenic steroids (EAAS). An open, non-randomized, interventional study in healthy volunteers ( $n = 8$ , 4 male, 4 female) was carried out with informed consent to obtain samples for steroid analysis as set out in Table 1. A total of 24 urine samples (Pre-administration/Day -3 = 8 samples, Post-administration/Day 0 and 3 = 16 samples) were collected.

For all samples, a 3 mL urine aliquot was prepared for GC-MS steroid profiling - internal standards were added before enzymatic hydrolysis, solid phase extraction and derivatisation with TMSI reagent (MSTFA/NH<sub>4</sub>I/ethanethiol) [6].

A Shimadzu GCMS-QP2010 Plus gas chromatograph mass spectrometer utilizing a HP Ultra-1 column (17 m x 0.20 mm i.d., 0.11 µm film thickness) in full scan mode (*m/z* 50 - 700) with single point calibration was used to estimate endogenous steroid metabolite concentrations. Subsequently, appropriate volumes of urine (5 - 20 mL) were prepared for GC-C-IRMS using previously published methodology [7]. Aliquots of urine were centrifuged before solid phase extraction on a C18 cartridge. The methanolic extract was evaporated then reconstituted in phosphate buffer for enzymatic hydrolysis. Liberated steroids were isolated by liquid-liquid extraction and acetylated before purification by high performance liquid chromatography. Three fractions were collected: F1 - etiocholanolone acetate (Et-Ac) and androsterone acetate (A-Ac), F2 - 5β-androstane diacetate (βαβ-Ac<sub>2</sub>) and 5α-androstane diacetate (ααβ-Ac<sub>2</sub>), and F3 - 5β-pregnanediol diacetate (PD-Ac<sub>2</sub>). Only F1 and F3 were processed owing to the presence of interfering pregnenolone metabolites in male Day 3 samples. After evaporation and reconstitution in cyclohexane, fractions were assessed by GC-MS to ascertain peak purity and to estimate analyte concentration, thereby allowing appropriate dilution for IRMS analysis. GC-C-IRMS instrumental conditions were as previously published [8].

Visit	1			2			3
Study Day	-3	-2	-1	0	1	2	3
Day of week	Fri	Sat	Sun	Mon	Tues	Wed	Thur
Clinical review	X			x			x
DHEA (100 mg)	X	X	X	X	X	X	
Men only - Pregnenolone (200 mg)				X	X	X	
Women only - Letrozole (2.5 mg)				X	X	X	
Hematology & biochemistry							X
Blood & urine sample	X			X			X

Table 1: Study protocol

## Results and Discussion

**GC-MS Steroid Profiling:** Highly elevated EAAS metabolite concentrations were observed in post-administration samples. 7/16 exceeded one or more WADA TD2004EAAS criteria for initiating investigation with GC-C-IRMS [9]. Using a population reference limits (99% RL) approach would have yielded a further 4 samples for IRMS [10].

**GC-C-IRMS Analysis:** δ<sup>13</sup>C values for Et, A and PD were obtained after correction for the added acetate moieties [11]. Pre- and post-administration samples were easily distinguished due to the marked depletion of δ<sup>13</sup>C for Et and A (Figure 1).

Δ<sup>13</sup>C values were calculated against PD (Figure 2). All pre-administration samples were less than the ASDTL decision limit of 4 ‰ (i.e. negative). Seven out of eight Day 0 samples were positive for one or more markers. Likewise, all female (Day 3, NO PREG) post-administration samples (4/4) were positive.

In contrast, all male (Day 3, PREG) post-administration samples were calculated negative for both markers. This was because the δ<sup>13</sup>C of the administered PREG matched closely to that of the administered DHEA (-30 ‰). Therefore the calculated Δ<sup>13</sup>C<sub>PD-Et or PD-A</sub> was reduced below 4 ‰, even approaching -4 ‰ for some measured Δ<sup>13</sup>C<sub>PD-Et</sub>. Because the dosage of PREG was very large, the PD did not suffer from any endogenous dilution, leading to a mean δ<sup>13</sup>C<sub>PD</sub> after PREG administration (*n* = 4) of -30.2 ± 0.5 ‰. In contrast, Et was recorded at somewhat less depleted values (-26.8 ± 0.6 ‰) for those samples, perhaps due to endogenous dilution or metabolic fractionation, leading to the unusual Δ<sup>13</sup>C<sub>PD-Et</sub>. However, for three out of four of these samples the corrected androsterone δ<sup>13</sup>C was less than -28 ‰, clearly indicating the administration of an exogenous steroid.

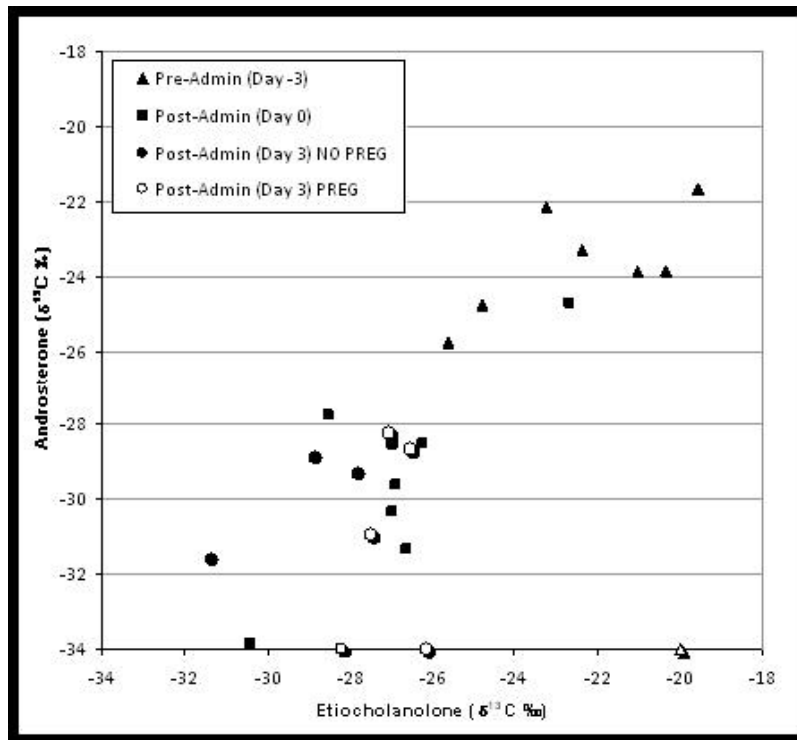


Figure 1: Pre (triangle) & Post-admin (square & circle)  $\delta^{13}\text{C}$  values for Et & A. Note: "white, with shadow" - No A values able to be determined for Subject 6.

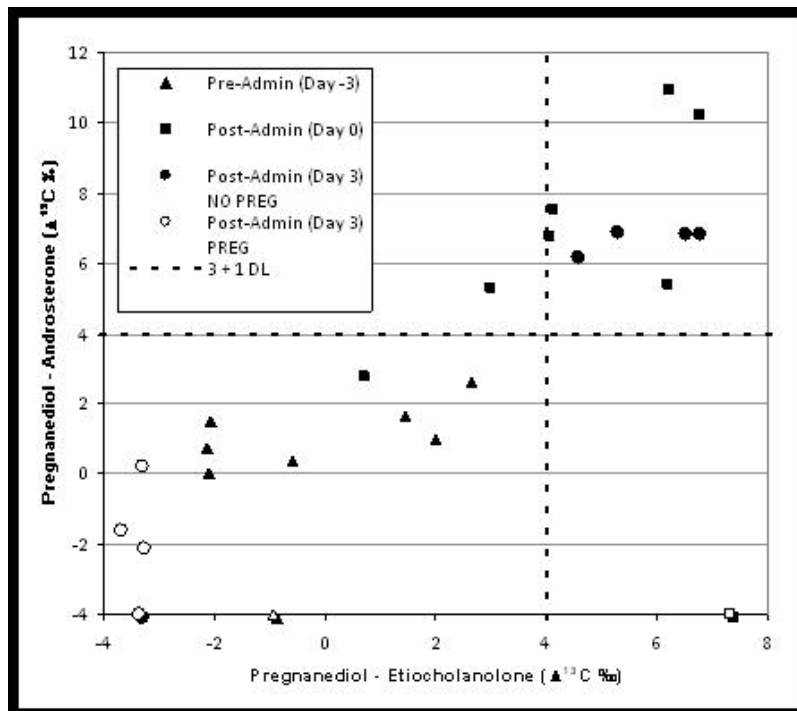


Figure 2: Pre (triangle) & Post-admin (square & circle)  $\Delta^{13}\text{C}$  values for Et & A relative to PD. Note: "white, with shadow" - No A values able to be determined for Subject 6.

## Conclusions

It was demonstrated that PREG *could* be used as a masking agent for the confirmation of EAAS abuse, as urine samples collected after the intake of DHEA did not fulfil WADA criteria for positivity when utilising PD as the ERC. However, the extremely large Et, A and PD concentrations found in prepared extracts clearly indicate a co-administration of DHEA with PREG, which is confirmed by the depleted  $\delta^{13}\text{C}$  values found for PD. To avoid such analytical problems during doping control procedures, an IRMS methodology that utilises multiple ERC from metabolically separate pathways should be used. As suggested previously, alternative ERC to PD may include  $5\alpha$ -androst-16-en-3 $\beta$ -ol [3,4] or 11 $\beta$ -hydroxyandrosterone [5].

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