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GC-C-IRMS analysis of endogenous reference compounds

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Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) has demonstrated great potential to detect endogenous steroid abuse in athletes [1]. Limitations exist, however, with our understanding and use of endogenous reference compounds (or ERC's) in carbon isotope ratio ($\delta^{13}C$) analysis. Interpretation of GC-C-IRMS results relies on the $\delta^{13}C$ equation (**1**) described by Harmon Craig [2] that is now calculated relative to the NBS-19 standard [3] via secondary CO_2 and/or alkane standards. Despite the fact that NBS-19 is not a physiological standard it is fit for the purpose of expressing the very small differences in steroid ^{13}C content (1‰ ≈ $\Delta^{13}C/^{12}C[0.0012]$) that form the distinction between endogenous and synthetic origins. The application of $\delta^{13}C$ analysis to doping control measures ^{13}C depletion relative to the natural ^{13}C content.

There is a need for doping control laboratories to appropriately determine ^{13}C depletion *in vivo*. One approach is to first consider the mathematics behind the $\delta^{13}C$ equation. Mathews [4] has proposed transformation to an amount of substance fraction thereby converting the δ-unit to the ratio of $^{13}C/^{12}C$ in the sample (**R**, Equation **2**). **R** may then be converted to the atom fraction of ^{13}C abundance (***F***, Equation **3**). Expressing the ^{13}C abundance as ***F*** still produces a number that is large compared to the difference in depletion measured among samples with GC-C-IRMS. Therefore, a δ-equivalent expressing ^{13}C depletion (***D***, Equation **4**) may be used by subtracting the natural ^{13}C abundance ($\boldsymbol{F_0}$) from the measured abundance ***F***. Natural abundance $\boldsymbol{F_0}$ will vary among individuals based upon dietary ^{13}C differences and may be different among genders and ethnic groups. Since $\boldsymbol{F_0}$ is an inter-individual variable, the determination of $\boldsymbol{F_0}$ for each measurement allows the definition of ^{13}C depletion below natural abundance to be established.

$$\delta^{13}C_{VPDB}(‰) = [R/R_{std} - 1] \times 1000 \quad (1)$$

$$R = (\delta^{13}C_{VPDB} / 1000 + 1)R_{std} \quad (2)$$

$$F = 13C/(13C + 12C) \quad (3)$$

$$D = F - F_0 \quad (4)$$

There are two methods by which to establish F_0 values:

- Reference $\delta^{13}C$ intervals assessing variables such as diet, gender and ethnicity.
- Determining the $\delta^{13}C$ value of an ERC.

Preliminary reference $\delta^{13}C$ intervals have been provided by our laboratory [5]. That work reported significant ($p < 0.0001$) differences in the $\delta^{13}C$ values obtained for etiocholanolone (Et; 5β-androstane-3α-ol-17-one), androsterone (A; 5α-androstane-3α-ol-17-one) and 11-ketoetiocholanolone (11keto-Et; 5β-androstane-3α-ol-11,17-dione) from Kenya, China, Australia and New Zealand. Upper reference intervals for Et and A were determined at -25.8‰ and -25.1‰ respectively. While these $\delta^{13}C$ profiling investigations involving urine samples collected from a further 10 countries are continuing, the latter method of $\delta^{13}C$ ERC determination for each sample analysed by GC-C-IRMS would have obvious advantages for laboratories without access to suitable reference intervals. Considering an ERC is a biochemical marker representing the isotopic signature of an athlete, the question is: which compound is most appropriate?

Like androgen metabolites, ERC's are derived from cholesterol. However in contrast to the anabolic nature of androgen biosynthesis, the corticosteroid pathway that produces ERC's is catabolic. Inactivation of steroid molecules generally occurs in the liver via oxido-reductive reactions. To render steroid molecules even more water-soluble, the majority of urinary steroid hormones are conjugated as their glucuronide form. The conversion of cholesterol to corticosteroids involves a complex series of reactions where a number of translocations of substrate must be made. First, to form pregnenolone (pregn-5-ene-3β-ol-20-one), cholesterol must pass into the mitochondria of adrenocortical cells before side-chain cleavage can occur by means of the desmolase reaction involving $C_{20,22}$-lyase as well as the 20α- and 22-hydroxylases [6]. This process is activated by adrenocorticotropic hormone (ACTH) secreted by the pituitary gland. Secondly, pregnenolone must pass out of the mitochondria and into the endoplasmic reticulum before 3β-hydroxylation with conjugation of the double bond can

occur to form progesterone (pregn-4-ene-3,20-dione). Cholesterol, pregnenolone and progesterone are the primary precursors for subsequent ERC biosynthesis.

Many ERC's have been proposed by laboratories for use in GC-C-IRMS analysis. The work of Becchi *et al.* [7] has seen many laboratories report the $\delta^{13}C$ value of pregnanediol (PD; 5β-pregnane-3α,20α-diol). PD is the major catabolic product of progesterone. It is formed by a process beginning with reduction of the $C_{4,5}$ double bond, primarily by 5β-reductase to form the 5β-pregnanedione metabolite, the rate-limiting step allowing 3α-hydroxylation of the 3-keto group to form 5β-pregnane-3α-ol-20-one. Finally, 20α-hydroxylation produces PD (Figure 1).

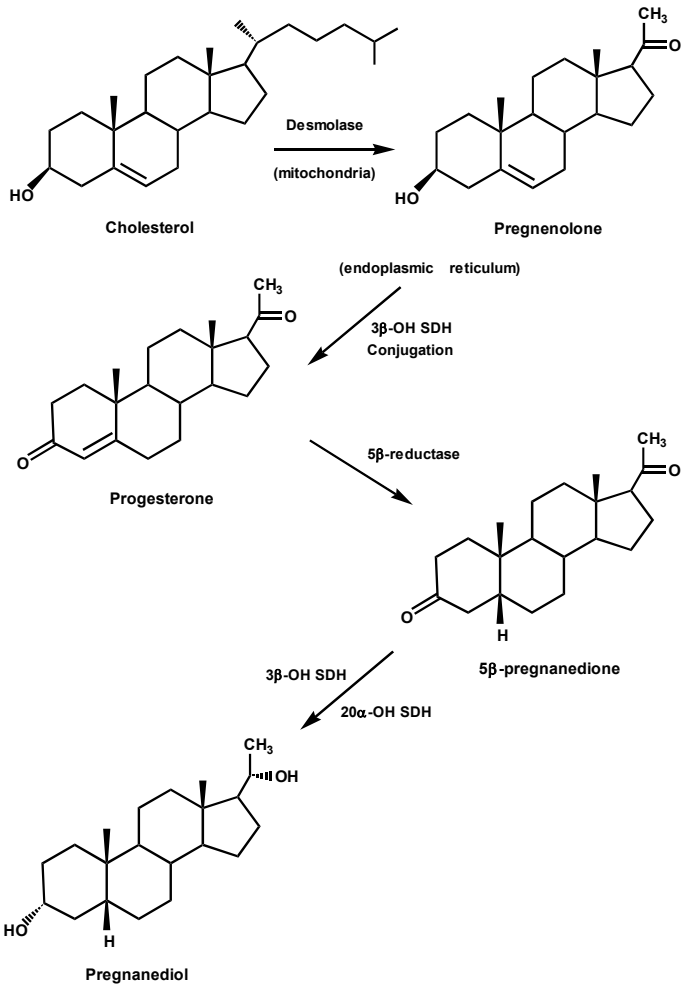

Figure 1: Biosynthesis of PD from cholesterol [6]

Another possible ERC is 11βOH-androsterone (11βOH-A; 5α-androstane-3α,11β-diol-17-one). This compound is produced from the cortisone-cortisol metabolic pathway, itself a branch from the production of pregnenolone and progesterone. Cortisone (pregn-4-ene-17α,21-diol-3,11,20-trione) and cortisol (pregn-4-ene-11β,17α,21-triol-3,20-dione) are inter-converted in the endoplasmic reticulum by 11β-hydroxysteroid dehydrogenase (11βOH-SDH). Side-chain cleavage is facilitated by $C_{17,20}$-lyase acting on both cortisone and cortisol to produce 11-ketoandrostenedione (androst-4-ene-3,11,17-trione) and 11βOH-androstenedione (androst-4-ene-11β-ol-3,17-dione) respectively that are also inter-converted by 11βOH-SDH. C_5-reduction is the rate-limiting step preceding 3α-hydroxylation of the latter intermediate to form 11βOH-A (5α-isomer) or 11βOH-Et (5β-isomer) as shown by Figure 2 [6].

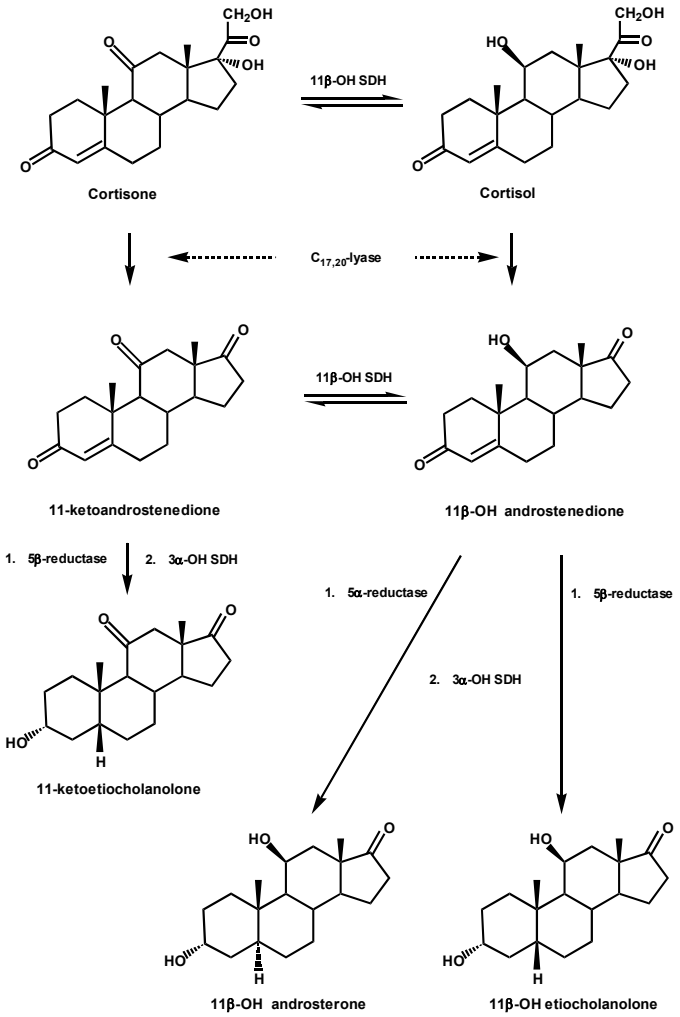

Figure 2: Biosynthesis of 11keto-Et, 11βOH-A and 11βOH-Et

Additionally, our laboratory has demonstrated the use of 11keto-Et as a suitable ERC for $\delta^{13}C$ analysis [8]. 11keto-Et is formed following 5β-reduction and subsequent 3α-hydroxylation of 11-ketoandrostenedione (Figure 2) [5]. Other reported ERC's include pregnanetriol (5β-pregnane-3α,17α,20α-triol) and 16(5α)-androstene-3β-ol [9]. The latter compound has demonstrated the potential to effectively identify doping violations in the case of oral pregnenolone being co-administered with an endogenous steroid [10]. The present study reports a survey of six measured ERC's: 11keto-Et, 11βOH-Et, 11βOH-A, cholesterol, PD and a coelution measurement (11keto-Et + PD). The reference $\delta^{13}C$ intervals of these ERC's and their associated $\Delta\delta^{13}C$ values relative to Et are used to propose the most effective ERC for doping control. The hypothesis was tested using a testosterone enanthate/placebo administration trial.

Experimental

To establish $\delta^{13}C$ reference intervals for the ERC measurements, a reference sample set was obtained from elite athlete volunteers with informed consent and ethics approval [11] who were located in Australia (n=37), New Zealand (n=38) and Malaysia (n=25). A double-blind testosterone enanthate/placebo administration study was also conducted with informed consent of 10 healthy male volunteers and ethics approval [12] for the intramuscular injection of 250 mg, once a week for six weeks. Steroid glucuronides were isolated from urine (10 mL) using PADII resin (Serva, Germany). The methanolic extract was evaporated to dryness before the addition of phosphate buffer (0.2 M, pH 7) to facilitate enzyme hydrolysis with β-Glucuronidase from *E.Coli* K12 (EC 3.1.2.31, Roche, Germany; 50 µL) for 1.5 hours in a water bath at 50˚C. The hydrolysate was adjusted to pH 9.8 with $K_2CO_3/KHCO_3$ buffer (20% w/v, 250 µL) before liquid-liquid extraction with diethyl ether (5 mL). The organic supernatant was evaporated to dryness before being reconstituted in acetonitrile/water (1:1, 100 µL). The extract was filtered through a 0.45 µm membrane before HPLC purification. A Hewlett-Packard 1090 LC system with automatic injection system coupled to a photodiode array detector (Palo Alto, CA, USA) and FOXY200 fraction collector (ISCO, USA) was used. The LC column, operated at 40˚C, was a LiChroCART® (125 mm x 4 mm x 5 µm) reverse phase C18 protected by a LiChroCART® (4 mm x 4 mm x 5 µm) C18 guard column (Merck, Germany). The injection volume was 90 µL. Gradient elution was performed at a flow rate

of 1.04 mL/min from the initial 80% water/20% acetonitrile to 100% acetonitrile at 20 min (4% acetonitrile/min) where it was held for 4 min. The column effluent was monitored at 345 nm, where β-trenbolone exhibits an absorption maximum. The fraction collector was programmed to dispense column effluent into separate test tubes at 40-second intervals from 7:40 to 14:20 and from 19:00 to 22:20, based on β-trenbolone having a retention time of 8:30. Table I provides the HPLC retention time and subsequent fraction collected for each analyte.

Table I: HPLC purification protocol

Fraction	Time (min:sec)	Analyte
1	7:40 – 8:20	
2	8:20 – 9:00	11βOH-Et
3	9:00 – 9:40	11βOH-A; 11keto-Et
4	9:40 – 10:20	T
5	10:20 – 11:00	E; βαβ-diol
6	11:00 – 11:40	ααβ-diol
7	11:40 – 12:20	Et
8	12:20 – 13:00	A
9	13:00 – 13:40	PD
10	13:40 – 14:20	
12	19:00 – 19:40	
13	19:40 – 20:20	Cholesterol
14	20:20 – 21:00	
15	21:00 – 21:40	5α-androstane-3β-ol (surrogate)
16	21:40 – 22:20	

Fractions 2, 3 and 15 were combined, and separately fractions 7, 8, 9 and 13 were combined before being evaporated to dryness. These two extracts were then reconstituted with ethyl acetate (50 μL) before being analysed by GC-C-IRMS using previously reported conditions [1]. Comparative statistical analysis was performed using Analyse-it® v1.73 for Microsoft® Excel while principal component analysis (PCA) was performed using Pirouette© 3.02 (Infometrix Inc).

Results and discussion

Profiling $\delta^{13}C$ intervals for the six ERC measurements are provided in order of ^{13}C depletion by Figure 3. Comparative analysis of $\delta^{13}C$ values showed PD to have the lowest ^{13}C content while displaying a parametric distribution, in relation to the other ERC measurements. The $\delta^{13}C$ range for PD (-19.4‰ to -23.4‰) had a mean of -21.4‰. The ^{13}C depletion trend was

confirmed by line plots reconstructed from PCA. Additionally, sample clustering based on country of origin was identified, with Australian and New Zealand samples being generally ^{13}C depleted in relation to those collected from Malaysia.

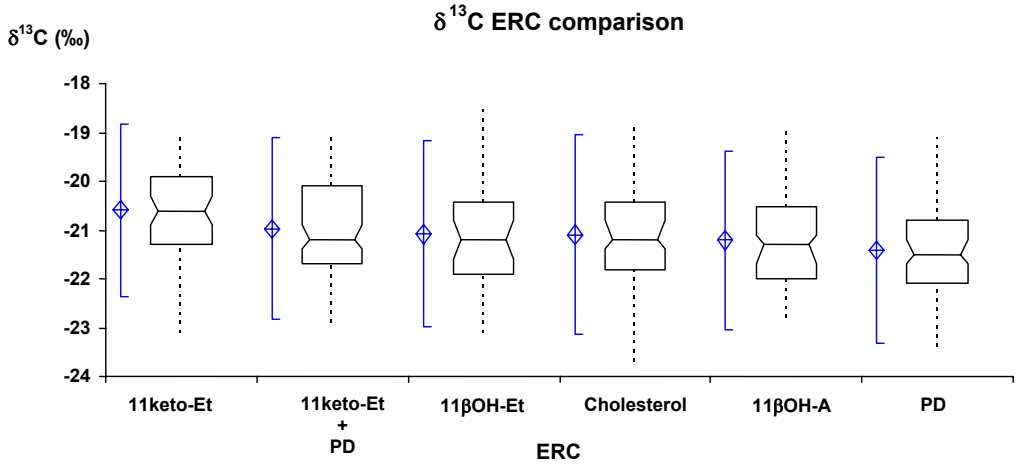

Figure 3: Profiling $\delta^{13}C$ intervals for ERC measurements (n=100). Dotted lines represent the range of $\delta^{13}C$ values. Brackets on the left-side of each distribution represent the distribution obtained from parametric transformation.

The application of $\Delta\delta^{13}C$ values to the confirmation of doping violations makes effective use of ERC's as normalisation factors to androgen metabolites. Figure 4 shows the $\Delta\delta^{13}C$ intervals of the measured ERC's relative to Et. Comparative analysis revealed similar distinction between ERC's based on their ^{13}C content. $\Delta\delta^{13}C$ values associated with PD were generally less than the other ERC measurements. Indeed, a skewed $\Delta\delta^{13}C$ PD distribution is observed towards lower values. No outliers were observed for PD, however the combined 11keto-Et/PD measurement had an outlier at a zero value, whilst 11βOH-A and 11βOH-Et had outliers at 4.0‰ and 4.5‰ respectively. Cholesterol displayed a statistically normal $\Delta\delta^{13}C$ distribution, although the occurrence of naturally higher $\Delta\delta^{13}C$ values greater than 4.0‰ with respect to Et was observed, most likely due to multiple sources of cholesterol in the body providing greater variance than observed for PD. PCA of $\Delta\delta^{13}C$ values revealed no inherent clustering of samples from the three countries, thereby demonstrating the $\Delta\delta^{13}C$ measurement as a useful means of accounting for variables such as diet.

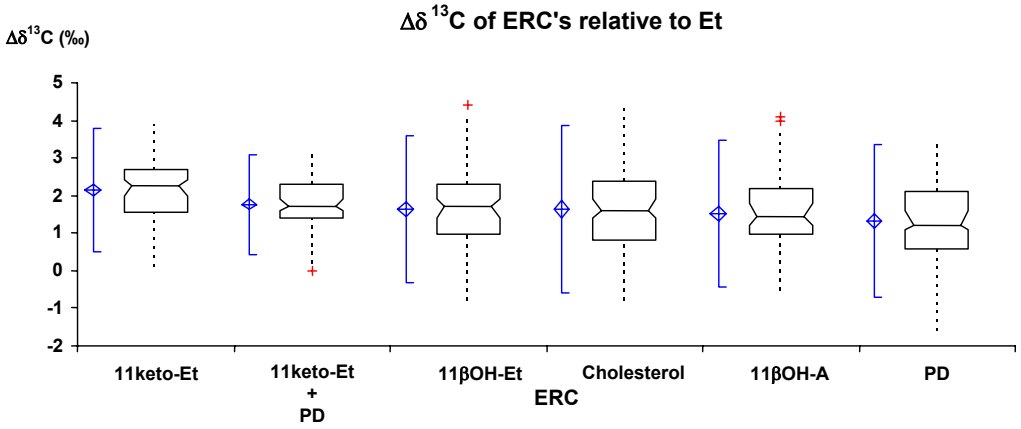

Figure 4: $\Delta\delta^{13}C$ reference intervals (n=100). Outliers are marked with +.

A testosterone enanthate/placebo administration was used to evaluate the influence of ERC on the interpretation of doping violations. The $\delta^{13}C$ value of testosterone hydrolysed from the enanthate ester was determined to be -29.9 ± 0.5‰. Figure 5 shows the initial and week 6 $\Delta\delta^{13}C$ values of each ERC measurement relative to Et (mean ± range) for the placebo group (n=5). PD is observed to have the lowest mean $\Delta\delta^{13}C$ value and associated upper range. The distinction between natural (baseline) and altered (week 6) states is made using $\Delta\delta^{13}C$ values illustrated by Figure 6. $\Delta\delta^{13}C$ values associated with each of the six ERC measurements exceeded 3.0‰, the confirmatory measure of endogenous steroid doping [13]. PD most effectively identifies testosterone enanthate administration with $\Delta\delta^{13}C$ values 4.2‰ higher than those observed at baseline and the greatest difference between $\Delta\delta^{13}C$ ranges.

A hypothetical situation examines $\Delta\delta^{13}C$ values from endogenous steroid administration based on ERC reference intervals. Table II provides the upper, mean and lower profiling $\Delta\delta^{13}C$ (Et) values for Et, 11keto-Et and PD in comparison to proposed $\Delta\delta^{13}C$ values expected from proportions of synthetic steroid ($\delta^{13}C$ = -29.9‰) administered. Issues associated with the choice of ERC and subsequent interpretation of doping cases can be clearly seen for mean $\delta^{13}C$ values of Et, 11keto-Et and PD. At 20% excretion of synthetic steroid, the $\Delta\delta^{13}C$ value of 11keto-Et and PD relative to Et would be 3.6‰ and 2.7‰ respectively. The former case would represent a doping violation, while the latter would not [13]. This can be interpreted in either of two ways: first that 11keto-Et provides greater retrospectivity to detect doping, or

second that PD provides the most conservative $\Delta\delta^{13}C$ values thereby maintaining legal defensibility of the measurement.

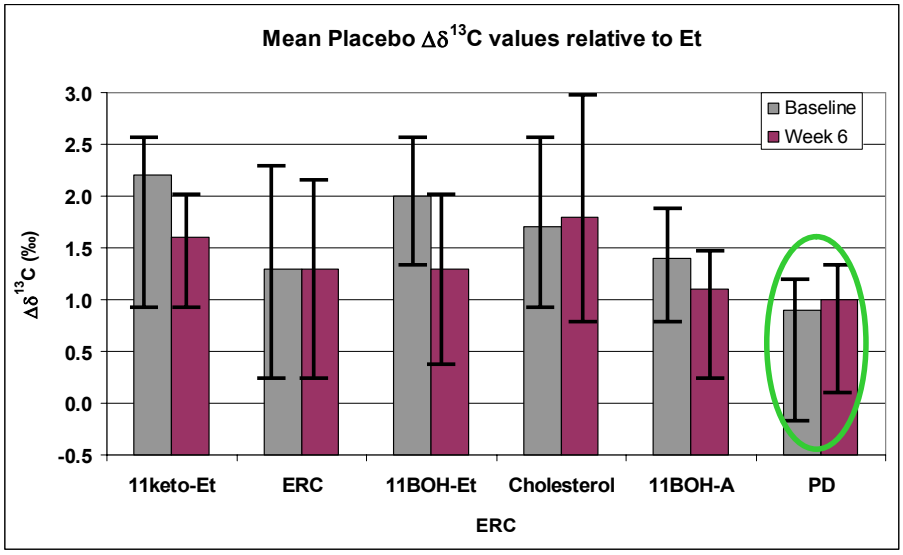

Figure 5: $\Delta\delta^{13}C$ values for placebo group (n=5) at baseline and week 6

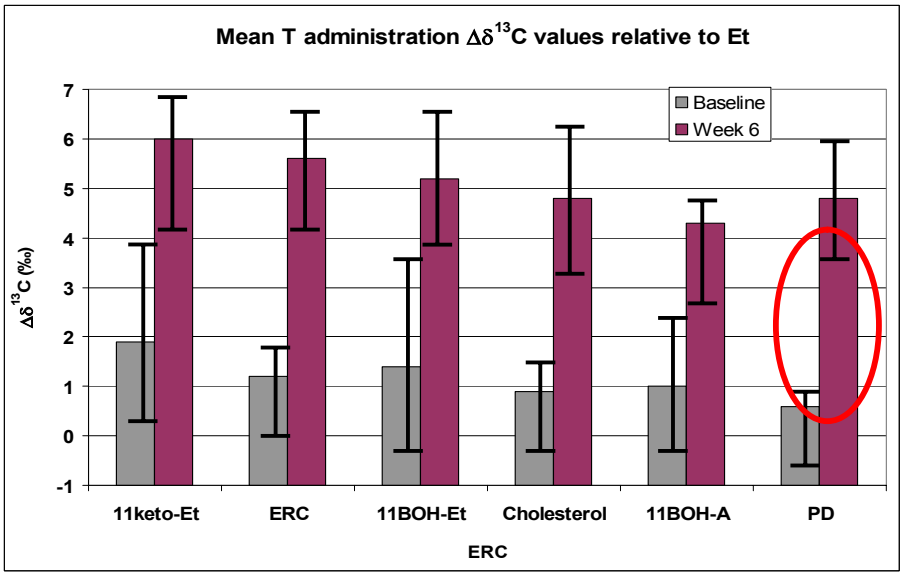

Figure 6: $\Delta\delta^{13}C$ values for testosterone administration group (n=5) at baseline and week 6

In summary, it is proposed that each ERC investigated in this study would be suitable for efficient GC-C-IRMS screening procedures with appropriate $\delta^{13}C$ reference intervals to make an informed decision concerning requirements for confirmation. PD, being most similar in ^{13}C content to the androgen metabolites should be used as the ERC for the confirmation of endogenous steroid abuse in athletes.

Table II: Hypothetical doping situation illustrating effect of ERC on $\Delta\delta^{13}C$ values with respect to Et

$\delta^{13}C$ Et (‰)	Exogenous %*	Calculated $\delta^{13}C$ Et (‰)	$\delta^{13}C$ 11-ketoEt (‰)	$\Delta\delta^{13}C$ (‰)	$\delta^{13}C$ PD (‰)	$\Delta\delta^{13}C$ (‰)
-24.4	80	-28.8	-23.1	5.7	-23.4	5.4
	50	-27.1		4.0		3.7
	20	-25.5		2.4		2.1
-22.8	80	-28.5	-20.6	7.9	-21.5	7.0
	50	-26.3		5.7		4.8
	20	-24.2		3.6		2.7
-20.5	80	-28.0	-19.0	9.0	-19.1	8.9
	50	-25.2		6.2		6.1
	20	-22.4		3.4		3.3

*Synthetic copy of an endogenous steroid has been administered with $\delta^{13}C$ = -29.9‰

% exogenous indicates proportion of synthetic material excreted in urine as Et

References

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