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# RECENT ADVANCES IN DOPING ANALYSIS (8)

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# Influence of pregnenolone administration on IRMS analysis

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## INTRODUCTION

Isotope Ratio Mass Spectrometry (IRMS) can be regarded as an attractive method in anti-doping control of testosterone misuse. It is based on the measurement of the isotopic ratio $\delta^{13}C^{0}/_{00}$ of testosterone (T) or of one of its main metabolites (TMs) such as androsterone (A), etiocholanolone (E), 5β-androstanediol (5β–ADiol) and 5α-androstanediol (5α–ADiol). However, in order to minimize the influence of inter-individual variability and any potential instrument calibration problems, it is recommended to relate the TMs isotopic values of to that of an Endogenous Reference Compounds (ERC). Therefore a relative criterion for positivity is defined as the ratio $\delta^{13}C^{0}/_{00}$ (TMs) / $\delta^{13}C^{0}/_{00}$ (ERC). From the last published results, pregnagnediol (PDiol) is recommended as the ERC (1)(2)(3).

This criterion assumes that PDiol only have an endogenous origin. However pregnenolone (P), the "grandmother" of hormones, is one of its possible precursor. P is a freely available compound in United States and is now associated with other " pro-hormone " such as 4-androstene 3,17 dione (4-ADione) in nutritional supplements. P is a precursor of DHEA and C21 steroids such as progesterone and PDiol. Hence it is expected that the $\delta^{13}C^{0}/_{00}$ value of PDiol is influenced by the use of P.

The aim of this study is to examine whether the administration of pregnenolone in conjunction with 4-androstene 3,17 dione introduces a bias in the relative criterion defined by the ratio $\delta^{13}C^{0}/_{00}$ (TMs) / $\delta^{13}C^{0}/_{00}$ (PDiol).

## EXPERIMENTAL

**Urine samples**

Four volunteers took orally 50 mg of P and 50 mg of 4-ADione. Fractionated urines were collected during at least 72 hours. Endogenous hormones were semi-quantified by GC/MS and isotopic ratios values of A, E, PDiol and 5β-ADiol were measured according to the procedure described by Aguilera et al.(2) with few modifications.

**Sample preparation :**

T/E ratio determination

2 ml of urine
|
Hydrolysis (β-glu) 55°C, 1 hour, pH 6.5
|
Adjust 9<pH<10
|
SPE C18 Wash $H_2O$/MeOH 90/10, Elute TBME
|
Derivatisation MSTFA/ITMS (1000/2)
|
GC/MS 2 µl split 1/20

IRMS procedure

8 to 16 ml of urine
|
SPE C18 Wash $H_2O$ - Elute MeOH
|
Hydrolysis (β-glu) 55°C, 1 hour, pH 6.5
|
SPE C18 Wash $CH_3CN$/ $H_2O$ 20/80, Wash $CH_3CN$/ $H_2O$ 35/65, Elute $CH_3CN$
|
Derivatisation Acetic anhydride + pyridine - 2 hour 80°C
|
SPE C18 Wash $CH_3CN$/ $H_2O$ 50/50, Elute $CH_3CN$/ $H_2O$ 75/25 (F1), Elute $CH_3CN$ (F2)
|
GC/C/IRMS

**Sample analysis :**

- T/E ratio determination

Semi-quantification (one point calibration) was performed by GC/MS (6970/5973, Hewlett Packard) in SIM mode on trimethylsilyl derivates (m/z 432 for T and E, m/z 434 for A and E, m/z 241 for 5β-ADiol and 5α-ADdiol, m/z 117 for PDiol and m/z 301 for the ISTD methyltestosterone).

- IRMS procedure

Monoacetylated steroids (A and E) were present in fraction (F1) and diacetylated steroids (5β–Adiol, PDiol) in fraction (F2). The internal standard (SI : 5α Androstanol 3β ol) was added before evaporation. The residues of diacetylated steroids were dissolved in 30 µl of $CH_3CN$ (100 µl for the fraction of keto-steroid). Isotopic ratio measurements were performed with a continuous flow isotopic mass spectrometer (Isoprime - Micromass). The separation was carried out using a DB-17MS column (25m, 0.25 mm ID, 0.25 µm). The oven temperature program was : after injection at 50°C a 1 min hold, then 30°C/min to 271°C and

0.6°C/min to 281°C (1 min hold), then 5°C/min to 300°C (5 min hold). Combustion was performed with a CuO furnace at 650 °C. Traces of water were eliminated by a cryogenic trap at –100°C ($N_2$ liq.). 3 µl of extracted were injected for the isotopic determination of 5β–Adiol and PDiol (1 µl for A et E) in splitless mode.

All isotopic values were given without correction of the derivatisation step.

## RESULTS

### Variation of T/E ratios :

The variation of T/E ratios over time for each subject is plotted in **figure 1**. After the administration of 50 mg of 4-Adione, a rapid increase of the T/E is observed in the first few hours. For each subject, a drop of the ratio below 6 is seen after 12 hours. For one subject (S1) the T/E ratio did not raise the threshold of 6. In this case the basline value T/E ratio was relatively low (0.5). These results are in good agreement with those reported by Van Eenoo P. et al. (4) or Uralets and Gillette (5).

N.B. : we have controlled that T/E ratio and the concentration of 5β–Adiol, A and E were not affected by the administration of P alone. Consequently, the evolution of T/E ratio can only be attributed to the administration of 4-ADione.

### Variation of $\delta^{13}C\ ^{0}/_{00}$ values (absolute criterion)

1- A shift of the isotopic ratios of A and E towards the exogenous values (-27 to –30) is observed with a maximum reached between 4 and 6 hours (**figure 2**). 12 hours after the injection of 4-Adione the presence of an exogenous administration was still detected by IRMS analysis for the four subjects although their T/E ratio had return to their baseline values.

2- In the first hours, isotopic ratios values of 5β–ADiol (**figure 3**) have a similar trend to those of A and E. But for subjects S2 and S3 their $\delta^{13}C\ ^{0}/_{00}$ (5β–Adiol) return slower to their baseline values. In those cases the window of detection of doping with 4-ADione could be extended.

N.B. : we checked that isotopic values of A, E, 5β–Adiol were not influenced by administration of P alone.

3- We have reported in **figure 3** a threshold of positivity that could be applied to absolute value of $\delta^{13}C^{0}/_{00}$ (5β–Adiol) in our laboratory. This threshold corresponds to the average value $\delta^{13}C^{0}/_{00}$ average value (27.9) + 3 times the standard deviation (0.88) calculated from 78 samples analysed at the LNDD during one year. Due to the inter-individual variation and instrument variabilities, if the endogenous value of $\delta^{13}C^{0}/_{00}$ of the subject is not know, as it is

the case in anti doping control, evidence of positivity can be difficult to establish on the criteria of absolute value of $\delta^{13}C^{0}/_{00}$.(5β–Adiol).

4- Concerning PDiol, isotopic values were greatly affected by the administration of P (**Figure 4**). The shift of $\delta^{13}C^{0}/_{00}$(PDiol) to the exogenous values are observed at least 72 hours after the administration of P (concentration of PDiol in urine was increased by a factor 3 to 6 in same time).

**Evolution of $\delta^{13}C^{0}/_{00}$ (5β–Adiol) / $\delta^{13}C^{0}/_{00}$ (PDiol) ratio (relative criterion)**

We reported in **figure 5** the $\delta^{13}C^{0}/_{00}$ (5β–Adiol) / $\delta^{13}C^{0}/_{00}$ (PDiol) threshold defined from our reference population (1.046 + 3 x 0.025 = 1.12) and the ratios calculated for the 4 subjects during this experiment. This threshold is in good agreement with those reported by Aguilera et al. (1.1) or Shackelton et al. (1.08). But due to the variation of PDiol $\delta^{13}C^{0}/_{00}$ values this criteria is no longer acceptable in this experiment. Regarding the $\delta^{13}C^{0}/_{00}$ (5β–Adiol) / $\delta^{13}C^{0}/_{00}$ (PDiol) ratios there is no clear evidence of abuse of a doping agent.

## CONCLUSION

These results indicate that even if IRMS analysis is a powerful tool for anti-doping control of testosterone and/or its precursors, it may give rise to false negative results due to the irrelevance of PDiol as the ERC. This is in particular the case when P was used in combination with others banned substance such as 4-ADione. When such a case is suspected from abnormal isotopic values of PDiol different solutions can be proposed. A different ERC can be used, for example cholesterol, DHEA... However standard clean-up procedure doesn't always allow to determine isotopic ratio of these compounds. Moreover, in the case of P administration $\delta^{13}C^{0}/_{00}$ value of DHEA will probably also be modified. It can also be proposed to not use the $\delta^{13}C^{0}/_{00}$ (TMs) / $\delta^{13}C^{0}/_{00}$ (ERC) criterion but absolute value of $\delta^{13}C^{0}/_{00}$ (TMs). In this case more care and prudence have to be take in order to avoid confusing interpretation on $\delta^{13}C^{0}/_{00}$ (TMs) values which are greatly influenced by instruments parameters (GC column, combustion efficiency, linearity and calibration of the instrument) and inter-individual variation (diet).

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(2) Aguilera R. et al., *J. Chrom. B.*, **1999**, 727, 95
(3) Ueki M. and Okano M., *Rapid. Commun. Mass Spectrom.*, **1999**, 13, 2237
(4) Van Eenoo P. et al. *Proceedings of the Manfred Donike Workshop, 16th Cologne Worshop on Dope Analysis*, **1998**, 6, 171
(5) Uralets V.P. and Gillette P.A., *J. Anal. Toxicol.*, **1999**, 23, 357

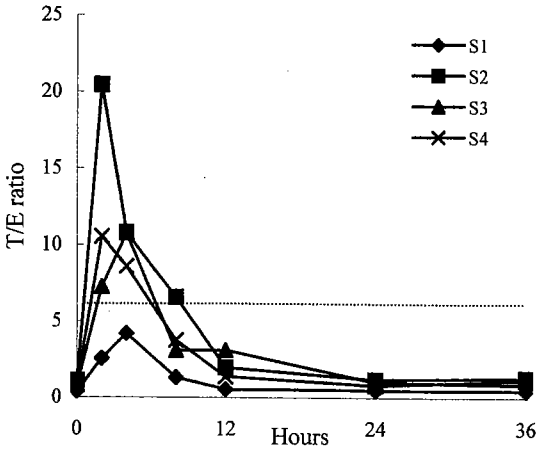


Figure 1

Figures 1- 5 : Variations of T/E ratios and $\delta^0/_{00}{}^{13}C$ values after administration of 50 mg of Pregnenolone (P) and 50 mg of 4-Androstene 3,17 dione (4-Adione).

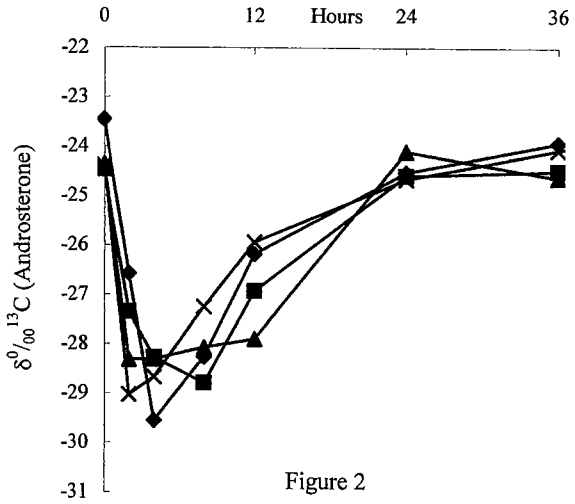


Figure 2

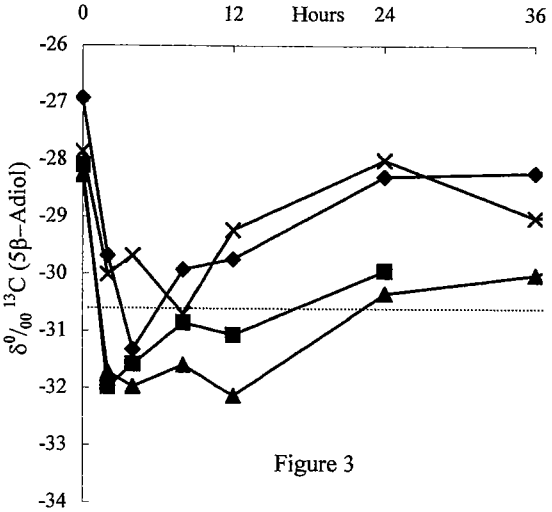


Figure 3

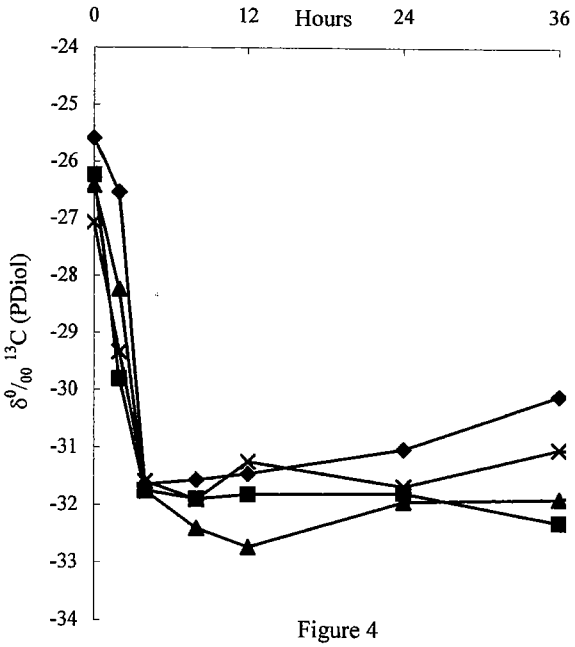


Figure 4

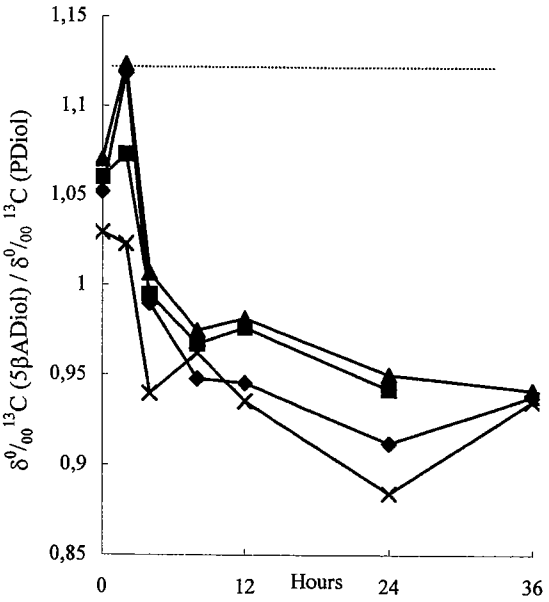


Figure 5