M. Abidi[1], S. Karkeni[1],N. Ben Hamida[2], A. Toumi[1]

Study of a case of WADA criteria shortage in the passage from GC/MS analysis to GC/IRMS one and investigation of an additional positivity criterion related to the second analysis

[1]Laboratoire National de Contrôle des Médicaments et de Dépistage du Dopage, 11 bis rue jebel lakhdar, 1006 Bab saâdoun, Tunis-Tunisie

[2]Laboratoire de chimie analytique et Electrochimie, Département de chimie, Faculté des Sciences de Tunis, 1060 Tunis, Tunisie

1- Introduction

Androstenedione (4-androstene-3,17-dione) is classified as an anabolic steroid as defined in the prohibited list published by WADA and such it is banned in most sports **[1].**

Many sports organizations prohibit the use of androstenedione and some test the urine of athletes for steroids; however the prohibition is difficult to enforce because consensus on the criteria for a positive case fixed by the WADA (TD2004EAAS) related to the passage from GC/MS analysis to GC/C/IRMS has not been reached after oral administration of androstenedione. Potential urinary markers of androstenedione administration include extremely high levels of testosterone, epitestosterone, DHT, androsterone, etiocholanolone, 5αdiol, 5βdiol and 6α-hydroxyandrostenedione and increase of T/E ratio.

The objectives of this study is to demonstrate that the criteria fixed by the WADA (TD2004EAAS) related to the passage from GC/MS analysis to GC/C/IRMS have not been fulfilled after oral administration of androstenedione.

This study suggests the investigation of additional criteria in GC/C/IRMS analysis based on the difference between $\delta^{13}C$ of androsterone, etiocholanolone and 5αdiol, 5βdiol respectively.

2-Excretion study

A single dose of 100 mg of androstenedione was administrated to a volunteer man. The urine samples were collected prior and after application (10 days) the excretion study is carried out to follow the concentration of the endogenous compounds and the determination of $\delta^{13}C$ by GC/MS and GC/CIRMS respectively.

3- Experimental

Sample preparation for GC/C/IRMS

To 10 ml of urine 200 µl of phosphate buffer (pH 6-7) were added to the urine sample and extracted with 5 ml of MTBE without hydrolysis, the organic layer was discarded however the aqueous one kept to the room temperature and the same procedure was applied (anabolics steroids screening) **[2]**, acetylation of the extract was carried out in 50 µl of pyridine and 50 µl of anhydride acetique at 65°C for 1 h incubation then evaporated to dryness under the nitrogen stream 2 ml of a mixture of water/acetonitril(50/50) were added to the residue.

The SPE column C18 (500 mg) was conditioned with 5 ml of distilled water and 5 ml of methanol. The sample was applied onto SPE columns, then eluted with 5 ml of acetonitril: H_2O (50:50) to recuperate the 11 keto-etiocholanolone which is called fraction 1. The fraction 2 containing androsterone and etiocholanolone was eluted with 5 ml acetonitrile: H_2O (75:25). The third one containing 16(5α)-androsten-3α-ol, pregnanediol, 5α diol and 5βdiol was eluted with 5 ml of acetonitrile **[3]**.

5α-androstane-3β-ol, (1 mg/ml in methanol) used as an external standard was added to each fraction 10, 30 and 10 µl respectively **[3]**. Then evaporated to dryness to be dissolved in 30, 100 and 30 µl of cyclohexane, respectively, and transferred to vials to be injected in GC/C/IRMS instrument.

GC/C/IRMS analysis: Isotopic Ratio measurements were performed with a continuous flow isotopic mass spectrometer (Isoprime- Micromass). The separation was carried out using HP-50 columns (30m, 0.25mm ID, 0.25 µm). The following oven program was applied 50°C(1min)-30°C/min to 271°C-0.6°C/min to 281°C (0min)-5°C/min to 300°C (5min) combustion was performed with a CuO furnace at 850°C. Traces of water were eliminated by a cryogenic trap at -100°C (N_2 liq).

4-Results and discussion

GC/MS : The screening results by GC/MS of the endogenous compounds of samples urines collected after administration of androstenedione we notice a rapid increase of testosterone and epitestosterone concentrations between 1 to 20 hours after the administration. Moreover the T/E ratio remains below 4 before and after 11 hours. The concentration of androsterone is observed with a maximum in 7 hours. 19 hours following administration, the concentration of androsterone still below 10000 ng/ml. For etiocholanolone the concentration did not reach the

threshold of 10000 ng/ml. The DHEA was not affected by the administration of androstenedione as shown in ***figure 1, 2, 3, 4, 5*** and ***6.***

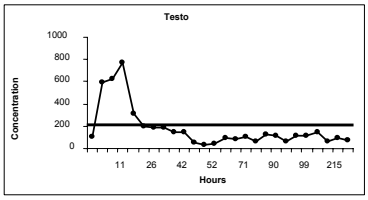

Fig.1

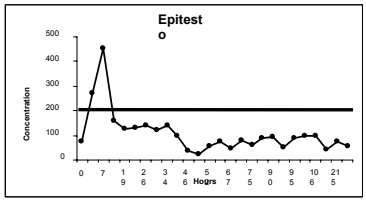

Fig.2

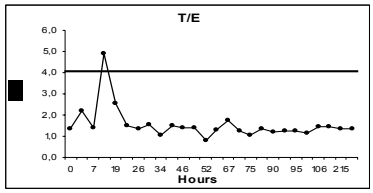

Fig.3

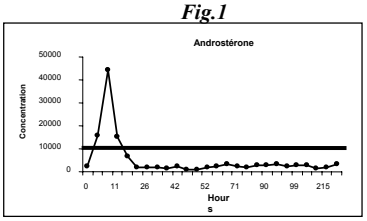

Fig.4

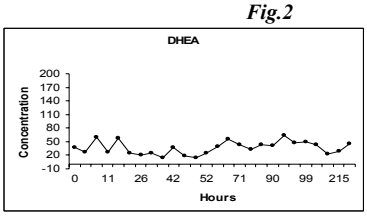

Fig.5

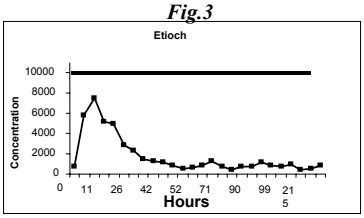

Fig.6

δ^{13}C-values following androstenedione administration: After ingestion of 100 mg of androstenedione, the δ^{13}C-values of androsterone, etiocholanolone, were significantly affected the values were – 29.20‰ and -27.88‰ respectively in the first two hours. Moreover the δ^{13}C-values difference between androsterone and ERC(16(5α)-androsten-3α-ol) still < -3 until 32 hours and more for the etiocholanolone until 58 hours ***fig.7*** and ***8***. We notice also that the δ^{13}C-values difference between androsterone and etiocholanolone is more significant in the space of time 42-75 hours.

The δ^{13}C-values difference between 5α diol, 5βdiol and Pdiol reaches the value -7 on 11 hours and decreases until 42 hours but still < -3 criteria fixed by WADA for 5α diol and until 69 hours for 5βdiol ***fig.9*** and ***10***.

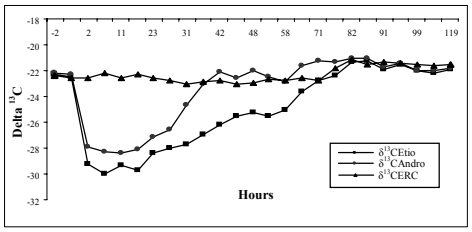

Fig.7

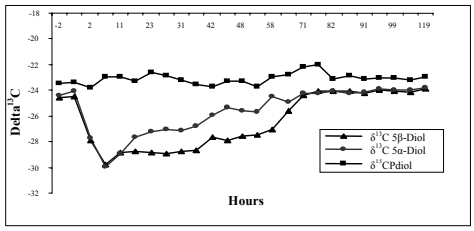

Fig.8

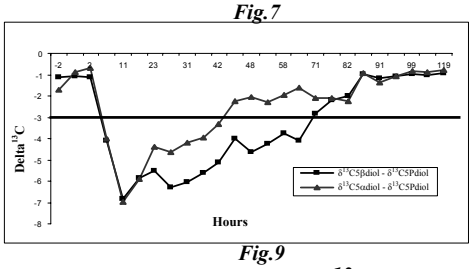

Fig.9

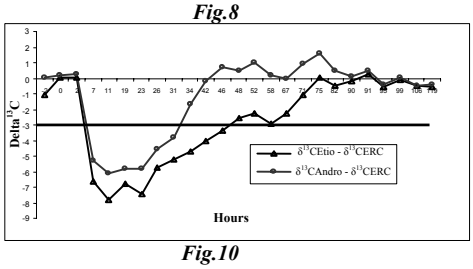

Fig.10

Study of the difference between δ^{13}C-value of Androsterone/Etiocholanolone and 5α diol/ 5βdiol after androstenedione administration: Normally the δ^{13}C-values of androsterone/etiocholanolone and 5α diol/5βdiol for healthy athletes are close. In this study, the δ^{13}C-values difference between androsterone and etiocholanolone is more pronounced

after 31 hours but insignificantly before and 26 hours after application of androstendione the same phenomena was observed in the third day of the excretion study as well as the $\delta^{13}C$-values differences between 5αdiol, 5βdiol ***fig.11*** and ***12***. This establishment can be helpful to interpret the positivity of the case based on there differences.

This additional criterion allows laboratories to avoid a false negative result by GC/C/IRMS analysis and to expect more comprehensive interpretation.

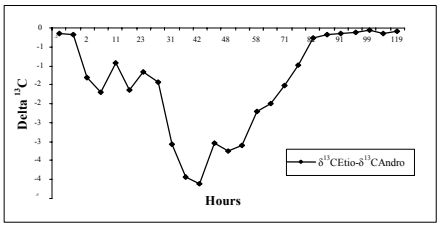

Fig.11

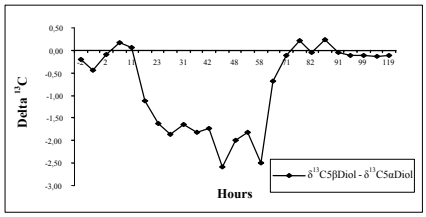

Fig.12

Conclusion

These results indicate that GC/C/RMS analysis has proven to be an efficient tool for anti-doping control of testosterone and/or its precursors. It is based on the calculation of the difference between $\delta^{13}C$ values of their metabolites and the ERC (16(5α)-androsten-3α-ol) and Pdiol. This analysis has yielded to the fact that the sample remains positive for 60 hours; however, the passage criteria from GC/MS analysis to the GC/C/IRMS one are no longer fulfilled in a 20 hours time following the oral administration of androstenedione.

This study has focused on the calculation of the differences between $\delta^{13}C$-values of androsterone and etiocholanolone as well as between 5αdiol and 5βdiol and proved that they have become more important in the case of androstenedione administration.

We suggest and taking account of these experiments results to adopt those differences as an additional criterion in GC/C/IRMS analysis.

This study can be extended and enriched by multiplying the number of volunteers and diversifying the population to establish a rule in order to harmonize the interpretation of isotopic measurement and to eliminate confusions and doubts.

References

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