Influence of *Saw Palmetto* and *Pygeum Africana* extracts on the urinary concentrations of anabolic androgenic steroids

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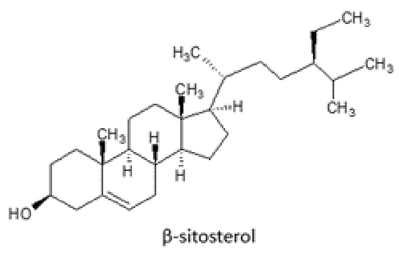
INTRODUCTION AND AIM

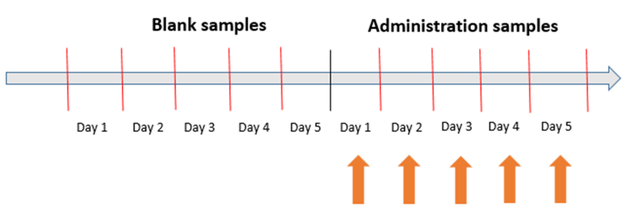
The detection of doping by «pseudo-endogenous steroids» (endogenous steroids when administered exogenously) is based on the longitudinal monitoring of six steroidal urinary markers (testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17α-diol (5αAdiol), 5β-androstane-3α,17α-diol (5βAdiol) and their relative ratios (T/E, A/T, A/Etio, 5αAdiol/ 5βAdiol, 5αAdiol/E) by the application of a Bayesian adaptive model that is able to predict the maximum variability for each marker based on the previous data to outline atypical results [1-3]. Although the introduction of the longitudinal steroid profile clearly improved the detection of the pseudo-endogenous steroid doping, it does not allow to gather any information on the occurence of atypical profiles due to the presence of endogenous (i. e. enzyme induction or inhibition, genetic polymorphism) or exogenous (i. e. banned drugs, masking agents, ethanol, bacterial contamination) confounding factors that could influence the urinary excretion of the described markers [4-5].

The aim of the present work is to evaluate the influence of the administration of a daily dose of nutritional supplement *(Saw palmetto* and *Pygeum africana* herbal extracts), containing β-sitosterol, on the urinary concentrations of a series of target steroids included in the Steroidal Module of the Athlete Biological Passport (ABP).

Natural plant extracts containing β-sitosterol, in particular from *Saw palmetto* and *Pygeum africana*, could be used in the treatment of lower urinary tract symptoms (LUST) associated with benign prostatic hyperplasia (BHP) and male androgenic alopecia because of their effects related to an inhibition of the enzyme 5α-reductase and/or with an inhibitory effect at level of androgens and estrogens receptors [6-7]. Since 5α-reductase inhibitors are routinely considered as possible confounding factors in cases of atypical steroid profiles [8], it seemed worthwhile to assess whether also the natural products containing *Saw palmetto* and *Pygeum africana* could influence the urinary levels of testosterone and its precursors and metabolites.



Excretion/administration study



To gather a good representation of the physiological levels of the markers of the Steroidal Module of the ABP, sample collection was performed for five days before starting the controlled excretion/administration study. After this period, three male volunteers took a dose of *Saw Palmetto* (320 mg) and *Pygeum africana* (100 mg) formulation orally, once a day for five consecutive days.

MATERIALS AND METHODS

Sample preparation

- 2 mL of urine
- 50 µL of internal standard
- 30 µL of β-glucuronidase from E. Coli
- 750 µL of phosphate buffer 0.8 M
- Hydrolysis for 1 hour at 55°C
- 500 µL of carbonate buffer 20% w/V
- L/L extraction with 5 mL of TBME
- Evaporation under nitrogen of the organic layer
- Derivatiztion with 50 µL of TMSI (30 min 75°C)
- Injection into GC-MS/MS system

Instrumental analysis

Gas chromatography conditions:
Column: HP 1 (l: 17 m; id: 0.2 mm; film 0.11 µm)
Carrier gas: Helium (He)
Injection temperature: 280°C
Injection mode: Split 1/20

Mass spectrometry conditions:
Source: electronic impact (70 eV)
Analyzer: Triple quadrupole
Analyzer temperature: 280°C
Acquisition mode: MRM (multiple reaction monitoring)

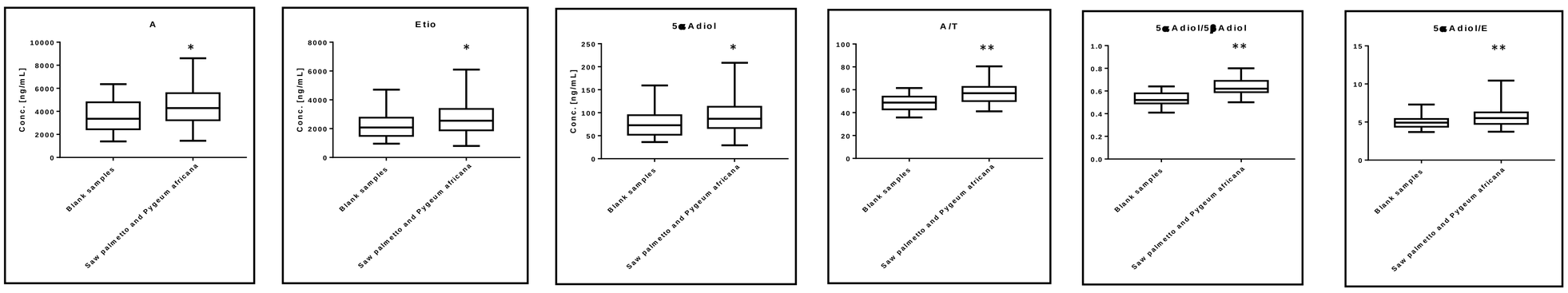
Selected tandem mass spectrometry for considered steroids

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
T bis-O-TMS	432	209; 196	20; 30
E bis-O-TMS	432	209; 196	20; 10
A bis-O-TMS	419; 434	329; 419	10; 10
Etio bis-O-TMS	419; 434	329; 419	10; 10
5αAdiol bis-O-TMS	256	185; 198	10; 10
5βAdiol bis-O-TMS	241; 256	159; 199	20; 10
T-*d3* bis-O-TMS	435	209; 196	20; 30
E-*d3* bis-O-TMS	435	209; 196	20; 30
A glucuronide-*d4* bis-O-TMS*	423	231; 333	20; 10
Etio-*d3* bis-O-TMS	424	221; 334	20; 10
5αAdiol-*d3* bis-O-TMS	259	188; 244	10; 5
5βAdiol-*d3* bis-O-TMS	246; 261	164; 199	20; 10
17α-methyltestosterone	301; 446	169; 301	20; 5

* Obtained after samples hydrolysis

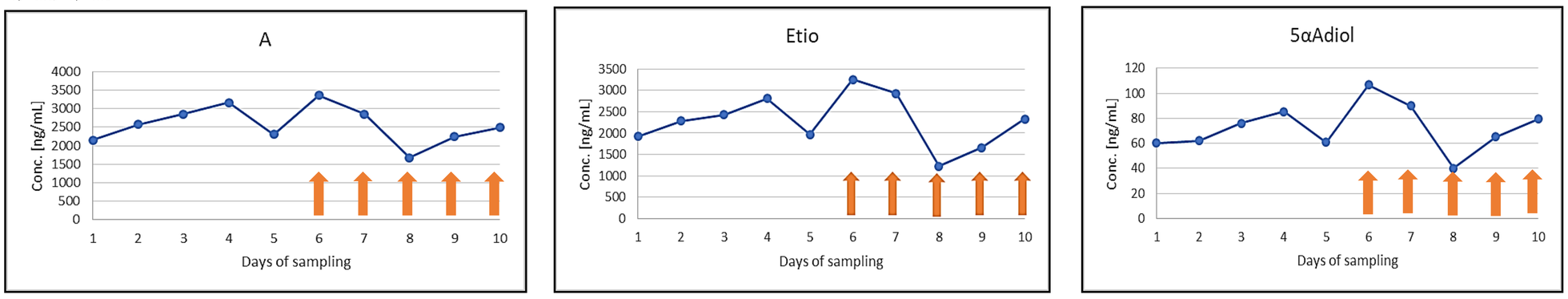
RESULTS

Effects of *Saw Palmetto* and *Pygeum africana* on the markers of the Steroidal Module of the ABP



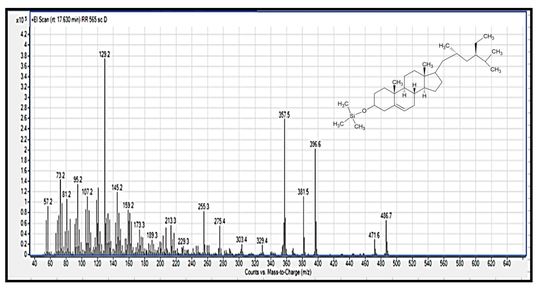
*: $p < 0.05$; **: $p < 0.01$

The intake of *Saw Palmetto* and *Pygeum africana* for five consecutive days is related to a statistically significant variation in the data distribution of A ($p < 0.05$), Etio ($p < 0.05$) 5αAdiol ($p < 0.05$) and of the ratios A/T ($p < 0.01$), 5αAdiol/5βAdiol ($p < 0.01$) and 5αAdiol/E ($p < 0.01$). The other parameters of the Steroidal Module of the ABP are not significantly affected. The data are expressed in terms of median, first and third quartiles and extreme values (box plots) for the samples collected before the administration (blank samples) and for the sample collected during the administration. The significance of the variation between the control and treatment urinary concentration groups was evaluated using a Student's t test. The p values less than 0.05 were considered statistically significant. Data reported refer to volunteer #2 but they are overlapping to those obtained in volunteer #1 and #3.

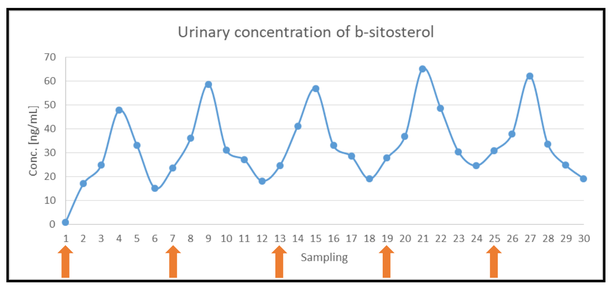


Longitudinal representation of the values of three parameters considered in the framework of the Steroidal Module of the ABP characterized by a statistically significant variation in the data distribution. Single data refer to the mean of the concentration values measured on the sample collected in the same day. The arrows indicate the days of administration. Data reported refer to volunteer #3 but they are overlapping to those obtained in volunteer #1 and #2.

Determination of the urinary concentration of β-sitosterol after the administration of *Saw palmetto* and *Pygeum africana* based supplement



GC-EI-MS spectrum of the β-sitosterol mono-TMS derivate. The selected MS/MS transition for the identification and quantification of the β-sitosterol in the urinary sample collected after the intake of *Saw palmetto* and *Pygeum africana* are:
m/z 1: 396/255 – 20 eV
m/z 2: 486/255 – 20 ev
m/z 3: 471/381 – 20 eV



Urinary excretion of b-sitosterol measured in the samples collected during the five days of administration of *Saw palmetto* and *Pygeum africana* based supplement. The arrows indicate the administration of one dose of the herbal supplement. Data reported refer to volunteer #2 but they are overlapping to those obtained in volunteer #1 and #3.

CONCLUSIONS

In this work we have highlighted the effect the intake of *Saw palmetto* and *Pygeum africana* herbal supplement on the parameters included in the Steroidal Module of the ABP. Our results have shown that the oral administration for five consecutive days provokes an alteration of the levels of some of the target steroids and of the corresponding concentration ratios, causing misinterpretations in the data collected in the framework of the ABP. Based on the above, the influence of *Saw palmetto* and *Pygeum africana* herbal supplements containing β-sitosterol on the analytical strategy currently adopted for the detection of pseudo-endogenous steroids doping should be carefully taken into account.

REFERENCES

[1] WADA Technical Document TD2018 EAAS.
[2] Athlete Biological Passport. Operating guidelines.
[3] Sottas PE et al. Biostatistic (2007).
[4] Mareck U et al. J Mass Spectrom (2008).
[5] Kuuranne T et al. Br J Sport Med (2014).
[6] Pais P et al. Res Rep Urol (2016).
[7] Buonocore et al. Arch Ital Urol Androl (2018).
[8] WADA Technical Document TD2018EAAS.

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