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Hydroxylated steroids stability.

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Abstract

Nowadays, testosterone (T) remains a highly abused anabolic androgenic steroid for the practice of doping. There are many causes that make its detection particularly complicated, since it is an endogenous compound and its concentration in spot urine samples is highly variable. Many approaches have been proposed for the detection of T misuse. From the classical T/E (reference population) to personalize the references, the individual became its own reference. To improve the urinary steroid profile one way is to extend its evaluation with the inclusion of new biomarkers, as proposed by Renterghem et al. [1] and Pozo et al. [2]. But little is know about the origin of these metabolites and on their stability in the different conditions of extraction.
In the first step of the study we have studied the stability of 25 hydroxylated steroids in different conditions: usual screening condition, soft hydrolysis and two aggressive hydrolysis conditions (acid hydrolysis with H_2SO_4/organic solvent and basic hydrolysis with KOH). Their behavior and their relationship with the new possible compounds formed were analyzed using two techniques GC-MS and LC-MS (TOF).
We could clearly demonstrate that, following different hydrolytic procedures, the hydroxylated steroid are stable as expected in screening or soft hydrolysis conditions, while in aggressive hydrolysis conditions the changes are very pronounced. New compounds are formed, and after a detailed characterization, it was found that the hydroxy group position on the steroid skeleton is important for the hydroxylated steroid stability and type of product formed.

Introduction

Nowadays, testosterone or its precursors remain highly abused anabolic androgenic steroids for the practice of doping. There are many causes that make their detection particularly complicated, since they are endogenous compounds and their concentration in spot urine samples is highly variable. To improve the classical urinary steroid profile, the inclusion of new biomarkers has been proposed [1,2] including hydroxylated steroids.
Moreover, almost no data are available about the origin of some of these metabolites (phase II metabolism) and on their stability in different conditions of extraction (usually followed for their analysis).
The aim of this research is to study the stability of 25 hydroxylated steroids under different hydrolysis conditions usually applied during screening and/or confirmation conditions to establish the steroid profiles.

Experimental

Hydrolysis

For the study 2 mL of synthetic urine [3] was used spiked with 0.2 mL of the 25 hydroxyl-steroid mix (Table 1). Five replicates for the five hydrolysis conditions were done; **NT**: non hydrolysis process; **H7:** The usual hydrolysis in the antidoping laboratory for the steroid analysis; **H9**: hydrolysis at 55 °C during one hour at pH = 9 using 0.5 mL of carbonate buffer (20%); **HB:** hydrolysis at 70 °C with 6N KOH during 15 min and **Strong Acidic**: hydrolysis at 70°C with a mix of EtAc/MetOH/H_2SO_4 (80:20:0.06) during 1 h.

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After the hydrolysis (except acidic) the samples were extracted as previously described [4]. After acidic hydrolysis and before the next step, the samples were neutralized with several drops of NH_4OH (30%) and evaporated under a N_2 stream. However, the hydroxylated steroids in this condition suffer a significant degradation (data not shown).

Principal Structure	Hydroxyl steroid position
Dehydroepiandrosterone (DHEA)	4β, 7α, 7β, 16α
Androstenedione (AED)	6α, 6β, 16α
Testosterone (T)	2α, 2β, 6α, 7α, 15α, 15β, 16α,16β
Androsterone (And)	6β, 11β, 16α, 16α–Epi
Etiocholanolone (Et)	6α, 6β, 11β, 15α, 16α, 16α–Epi

Table 1: The 25 hydroxy-steroid mix.

GC-MS Analysis

The metabolites extracted were analyzed as TMS derivatives, as previously described [4].

LC-MS (TOF) Analysis

Additionally, the steroids extracted were redissolved in 50 µL of ACN in H_2O (15%).
Scan mode method (100 to 1000 m/z) was carried out using an Agilent 6520 Accurate-Mass Q-TOF (Palo Alto, USA), equipped with a Dual Spray Electrospray Source (ESI+) interfaced to a LC system (Agilent, 1200 series, Palo Alto, USA).
The LC separation was performed using a Discovery C18 column (150 x 2.1 mm x 5 µm, Supelco), at a flow rate of 250 µL/min. Water and acetonitrile both with formic acid (0.1%) were used as mobile phase solvents. A linear gradient program was used: 15 % (0 min) percent of organic solvent, 15 % (8 min), 30% (18 min), 40 % (18.01 min), 40% (22 min), 50% (22.01 min), 50% (25 min), 100% (25.01 min), 100 % (30 min).

Data analysis

The data were imported into SIMCA-P+ 12 (Umetrics, Umea, Sweden) for statistical data analysis. Principal component analysis (PCA) was performed with mean centering, logarithm transformation and Pareto scaling as data pre-treatment. The control group (**C**) is the initial mixture of standards derivatized without applying any hydrolytic process.

Results and Discussion

Mix Study using PCA

The PCA analysis (Figure 1A) shows a clear difference between the group of **SB** and the other samples, and demonstrated that a basic hydrolysis has an effect over the hydroxyl steroid. The other groups are not located in the same sector of the controls (**C**), because of the extraction procedure. The closer cluster is the **H7** group. If we see the loading plot (Figure 1B) there are three types of groups of steroids after the hydrolysis process: (A) the hydrolysis and extraction do not have a great influence, (B) the hydrolysis does not have any effect over the metabolites, but the extraction yes and (C) the hydrolysis and extraction influence the response of steroids.

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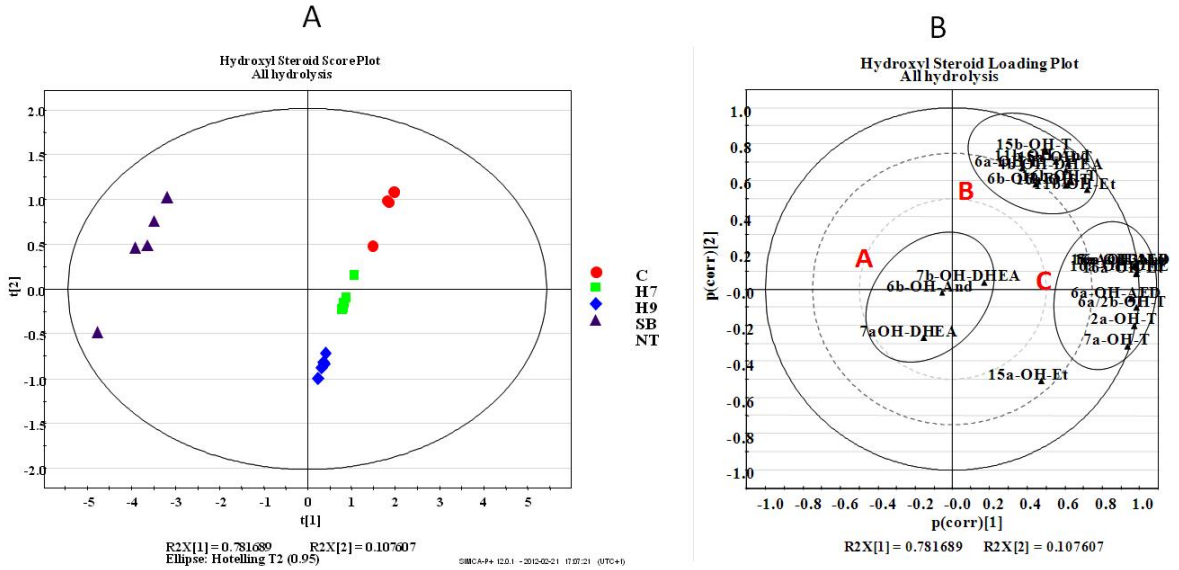

Figure 1: Score (A) and loading plot (B) of PCA analysis of hydrolysis stability.

The group C and 15a-OH-Etiocholanolone were submitted to strong basic conditions and were studied separately and analysis by LC-MS(TOF). In Table 2 are summarized the characteristic these compounds. From the table is noticeable that the hydrolysis products depend on the hydroxyl group position. The principal products formed could be classified in isobaric, oxidation and dehydration compounds. The study of two metabolites and their products will be shown.

Compound	Standard		Isobaric		Oxidation		Dehydration (Type I/II)	
	RRt	[M+H]+	RRt	[M+H]+	RRt	[M+H]+	RRt	[M+H]+
7a -OH-T	0.65	305.2101					0.91	287.1978 (I)
2a -OH-T	0.84	305.1996			0.89	303.1875		
2b-OH-T	0.63	305.2095	0.69	305.2120			0.95	289.2172 (II)
6a -OH-T	0.57	305.2091	0.69	305.2119			0.95	289.2190 (II)
6a -OH-AED	0.71	303.1911	0.79	303.1972			0.99	287.2025 (II)
6b-OH-AED	0.73	303.1946	0.79	303.1978			0.99	287.2012 (II)
16a -OH-DHEA	0.78	305(287.2023)	0.71	305(287.2023)	0.78	303.1982		
16a -OH-AED	0.81	303.1941	0.75	303.1967				
16a -OH-Eti	0.94	307(289.2177)	0.89	307(289.2183)	0.92	305.2127		
16a -OH-And	0.94	307(289.2160)	0.89	307(289.2183)	0.92	305.2136		
16a -OH-EpiAnd	0.82	307(289.2172)	0.77	307(289.2184)	0.83	305.2125		
16a -OH-EpiEti	0.86	307(289.2173)	0.80	307(289.2184)	0.85	305.2131		
15a -OH-Eti	0.88	307(289.2150)					1.06	289.2166 (I)

Table 2: Characterisitc summary of the group C and 15a-OH-Etiocholanolone studies separately, LC-MS (TOF) technique.

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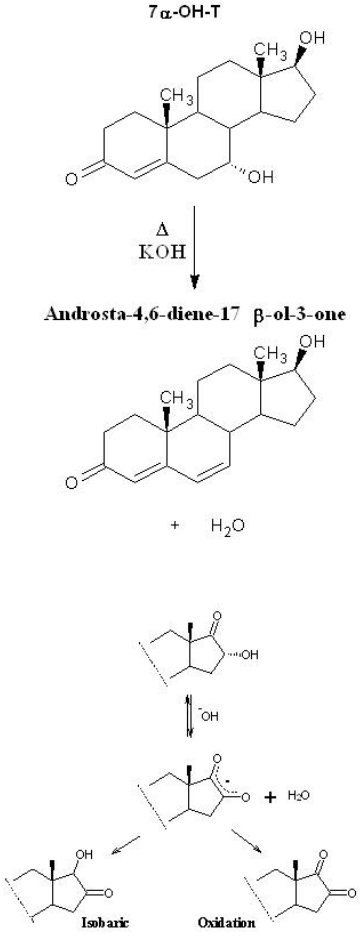
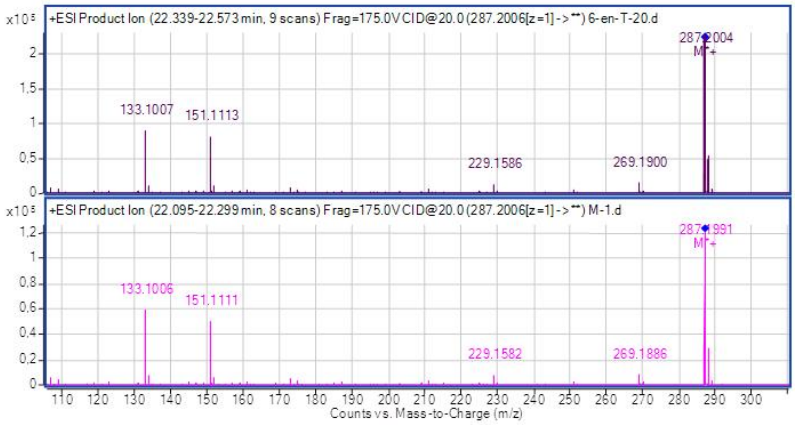
7a-OH-T

Is the only compound that undergoes a total dehydration. The product compound has a molecular formula $C_{19}H_{26}O_2$ with a mass difference of 4.9 ppm, estimate from the relative masses of the molecular ion detected in the TOF. This compounds is characterized by the same relative retention time (trr = 0.91) and mass spectral of the **4,6-androstadiene-17b-ol-3-one** (reference material), see Figure 2.



Compounds	Parameters				
	Molecular Formula	Theoretical [M+H]+	trr	Measured [M+H]+	Diff (ppm)
7a-OH-T	$C_{19}H_{28}O_3$	305.2111	0.65	305.2101	3.2
Dehydratation	$C_{19}H_{26}O_2$	287.2006	0.91	287.1991	5.2
Ref.material	$C_{19}H_{26}O_2$	287.2006	0.91	287.2004	0.6



Compounds	Parameters				
	Molecular Formula	Theoretical [M+H]+-H2O	trr	Measured [M+H]+-H2O	Diff (ppm)
16a-OH-Eti	$C_{19}H_{30}O_3$	289.2162	0.94	289.2177	5.1
Isobaric	$C_{19}H_{30}O_3$	289.2162	0.89	289.2183	7.4
Oxidation	$C_{19}H_{28}O_3$	287.2006	0.92	287.2018	4.2

Figure 2: Reaction scheme proposed and some characteristics of 7a-OH-T and 16a-OH-Et hydrolysis

16a-OH-Eti

The hydrolysis of C-16 OH-Steroids causes the formation of a negative ion by resonance, for that it is necessary a carbonyl group in b position (Figure 2). The successive step of the reaction is the formation of two new structures, an oxidized molecule and isobaric one. This would explain the experimental results.

Conclusions

The hydroxylated steroid are stable as expected in screening or weak hydrolysis conditions, while in strong hydrolysis conditions the changes are very pronounced. New compounds were formed, and after a detailed characterization was found that the hydroxy group position on the steroid skeleton is important for the hydroxylated steroid stability and type of product formed.

References

1.- Van Renterghem P., Van Eenoo P., Van Thuyne W., Geyer H., Schänzer W., Delbeke F.T. Validation of an extended method for the detection of the misuse of endogenous steroids in sports, including new hydroxylated metabolites. Journal of Chromatography B, 876 (**2008**) 225–235;

2.- Pozo O.J., Marcos J., Ventura R., Fabregat A., Segura J. Testosterone metabolism revisited: discovery of new metabolites. Analytical and Bioanalytical Chemistry 398(4) (**2010**) 1759-1770;

3.- Leinonen A, Kuuranne, T., Moisander, T., Rautava, K., Artificial urine as sample matrix for calibrators and quality controls in determination of testosterone to epitestosterone ratio. In: Schänzer W, Geyer, H., Gotzmann, A., Mareck, U., editor. Recent Advances in Doping Analysis, vol 15. Köln (Germany): Sportverlag Straus (**2007)** 401-404.

4.- Mazzarino M., Abate MG., Alocci R., Rossi F., Stinchelli R., de la Torre X., Botre F. Urine stability and steroid profile: Towards a screening index of urine sample degradation for anti-doping purpose. Analytica Chimica Acta 683 (**2011**) 221–226.

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