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Profiling of endogenous steroids by UHPLC-QTOF-MS^E

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Abstract

Testosterone (T) is an anabolic androgenic steroid (AAS) that is widely used as doping agent. The World Anti-Doping Agency (WADA) has determined criteria to consider for T abuse, based on the steroid profile, including precursor, epimer and metabolites of T. These steroids are extensively metabolized in urine as phase II metabolites (glucuronide and sulfate conjugates), but the direct quantification of these intact metabolites that may help to gather worthy information on endogenous T metabolism is still challenging.
In this study, a method coupling ultra-high pressure liquid chromatography (UHPLC) to hybrid quadrupole time-of-flight (QTOF) mass spectrometry was developed and validated. UHPLC offers high chromatographic performance by using columns packed with small particles (i.e. sub-2µm), and QTOF mass analyzer enables exact mass determination on molecular and fragment ions over the entire selected mass range. After a sample preparation by solid phase extraction (SPE), a highly selective chromatographic separation was performed with baseline resolution between pairs of isomers. The analytes were detected in the electrospray negative mode and 2 functions were acquired simultaneously in the MS^E mode. This allowed the quantification of the investigated metabolites by assessing the molecular ion obtained in the first function at low collision energy (5 eV), while the second function acquired at ramped collision energy (5 to 70 eV) afforded a rich fragmentation pattern helping the identification of T analogs.
This development revealed the promising opportunity to supply a broader steroid profiling including an extensive monitoring of endogenous metabolites (steroidomics). Chemometric tools were used to highlight biomarkers of interest and underlined the ability of this analytical strategy to extend the detection window of T doping.

Introduction

The misuse of testosterone (T) is screened by determining the urinary steroid profile, i.e. T, precursor and metabolites which is generally performed by GC-MS after hydrolysis of the glucuronide part and derivatization. The T to epitestosterone (E) ratio (T/E) ≥ 4 is considered as the most sensitive parameter for suspicion of T abuse [1]. However, as these molecules are extensively metabolized in urine as phase II metabolites (glucuronide and sulfate conjugates), the information, provided by the phase II metabolism, is partially lost during the hydrolysis step. The direct quantification of intact steroid glucuronides by means of LC-MS is expected to provide a deeper insight into the glucuronide- (G) and sulfo- (S) conjugation of T, epitestosterone (E), dehydroepiandrosterone (DHEA), androsterone (A) and etiocholanolone (Etio).
Metabolomics using ultra-high pressure LC (UHPLC) has given new opportunity for targeted and untargeted analysis. Indeed, this technique promises high chromatographic resolution with increased peak capacity in a reasonable analysis time. When coupled to high-resolution mass spectrometer, such as a quadrupole time-of-flight (QTOF) device, MS^E acquisition can be performed, allowing accurate mass determination of parent and fragment ions in a single run [2,3]. Untargeted metabolomics constitute an interesting approach to reveal biomarkers related to a physiological response and to differentiate biochemical phenotypes. Furthermore, chemometric tools are mandatory to deal with the wealth of information and data processing [4].
After UHPLC-QTOF-MS^E analysis and data treatment, the most relevant candidate biomarkers were structurally identified and could be included in the Athlete Steroidal Passport.

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Experimental

Population study and sample collection

A clinical trial was realized based on the administration of T undecanoate tablets [5]. Multiple oral doses were administered during one month to volunteers who belonged either to the placebo group (300 mg of mannitol), or to the testosterone group (80 mg of T undecanoate and 115 mg of mannitol). Blood and urine samples were collected, and for each volunteer, 6 urine samples were selected among the samples collected during this clinical trial. A basal state at t00 was compared to a kinetic follow-up of 24 h (t01-t04) and a return to basal situation at t05 corresponding to more than 200 h after the last pill intake.

Sample preparation

A selective extraction of glucuronide-conjugated (G) and sulfo-conjugated (S) anabolic androgenic steroids (AAS) was applied to all urine samples. A solid phase extraction (SPE) was performed with Oasis HLB (Waters, Milford, MA, USA) cartridges 30 mg (30 µm particle size). The loading solution was obtained by diluting 1 mL of urine with 1 mL of a 2% formic acid (FA, aqueous solution). Two washing steps followed with 1 mL 2% FA (aqueous solution) and 1 mL 5% ammonium hydroxide (aqueous solution) containing 10% methanol. The analytes were eluted with 500 µL solution of methanol /water (40/60). The elute phase was evaporated to dryness under air stream and reconstituted in 100 µL solution of ACN /water (30/70).

UHPLC-QTOF-MS^E

Sample analyses were performed with an Acquity UPLC system (Waters, MA, USA) coupled with a Micromass-Q-TOF Premier mass spectrometer (Waters) equipped with an electrospray ionisation (ESI) source. Separations were carried out on Acquity UPLC columns (BEH C18 150 x 2.1 mm, 1.7 µm) at 25°C and 300 µL/min. The mobile phase was (A) 0.1% FA in water, and (B) 0.1% FA in ACN. A chromatographic gradient was started linearly from 5% to 37% B over 25 min. The injection volume was fixed at 20 µL in the full loop mode and samples were maintained at 4°C in the autosampler.
MS conditions in negative mode: capillary voltage 2400 V, cone voltage 50 V, source temperature 120°C, desolvation temperature 360°C, cone gas flow 10 L/h, and desolvation gas flow of 800 L/h. Detection was performed in the *m/z* range 95–1000 in centroid mode with a scan time of 0.2 s and an inter-scan delay of 0.02 s. The QTOF mass spectrometer was operated in wide-pass quadrupole mode with a low collision energy set at 5 eV in the first function and a collision energy ramp from 5 to 70 eV was applied in the second function (MS^E mode).
A solution of Leucine-Enkephalin (Sigma-Aldrich, Buchs, Switzerland) at 2 µg/mL was infused through the Lock Spray™ probe at a flow rate of 5 µL/min for the Dynamic Range Enhancement (DRE) lockmass. Data acquisition and instrument control were performed using the MassLynx Software (Waters).

Data analysis

N-PLS-DA models were performed under the MATLAB® 7 environment (The MathWorks, Natick, USA) with routines implemented in the N-Way Toolbox (version 3.1) [6]. For each model, a leave-one-out cross-validation was performed to assess the model fit and indices of accuracy, sensitivity and specificity were computed to measure classification performance. Model validity was verified using permutation tests (Y-scrambling). A thorough description of the method can be found in [2,7].

Results and Discussion

Method Development

UHPLC-QTOF-MS^E allowed sensitive and selective detection of endogenous AAS on a wide range of concentration (1 ng/mL to 8 µg/mL). The sample preparation by SPE HLB cartridges was optimized to obtain a pre-concentration factor of 10. For the chromatographic conditions, a low gradient was required to separate the 10 targeted analytes and to ensure the selectivity of the method for urine matrix. The extracted ions of glucuronide-conjugated steroids are presented in Figure 1A.
The efficient separation of UHPLC and accurate mass measurement allowed for the resolution of the compounds. However, for the compound DHEA-glucuronide, higher selectivity was mandatory due to three unknown, co-eluting isomers. As presented in Figure 1B, by setting the temperature to 25°C, baseline separation could be obtained for the isomers. The QTOF-MS^E acquisition mode was selected, as it allowed obtaining a function at low collision energy (5 eV) and a function at ramped collision energy (5-70 eV) in a single analysis. The first function permitted to monitor the parent ion with high intensity, used as the quantitative ion. The ramped collision energy applied in the second function provided structural

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information through fragmentation pattern obtained without selecting the precursor ion.

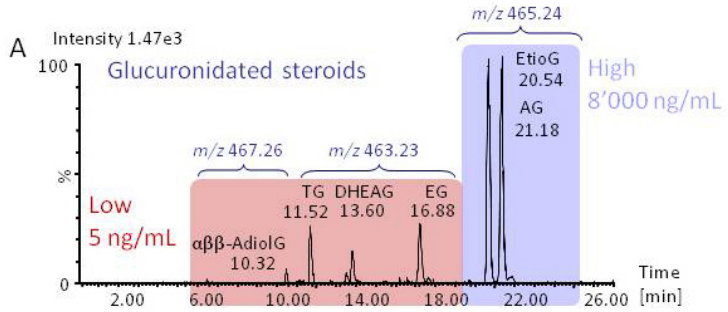

Figure 1A: Extracted ion chromatograms (XIC) glucuronide-conjugated steroids at m/z 467.26, 463.23 and 465.24 ± 0.05 Da

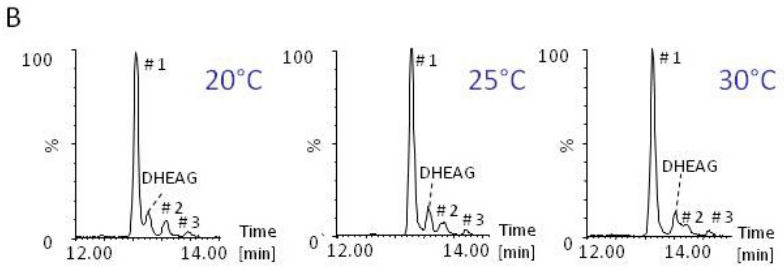

Figure 1B: XIC for the trace m/z 463.23 ± 0.05 Da with the column temperature maintained at 20, 25 and 30 °C. Peak #1, #2, #3 are endogenous isomers of DHEA-glucuronide (DHEAG)

Validation

The method was validated according to the guidelines and protocols from the Internal Standard for Laboratory [8], and the Food and Drug Administration [9]. The validation parameters consisted of selectivity, matrix effects, carry-over and stability. A quantitative validation was performed based on the International Conference on Harmonization protocol [10].
Trueness, repeatability, intermediate precision, lower limit of quantification (LLOQ) and limit of detection (LOD) are presented in **Table 1** and were found appropriate for application to clinical study samples.

Application to clinical study samples

Targeted Profile

Based on the quantitative results obtained for the 10 targeted steroids, the most sensitive information was obtained through the T/E ratio. T/E values for each subject either from the placebo or from the T undecanoate group are presented in **Table 2**. As expected, all of the T/E ratios from the placebo class were below 4 and considered as negative. However, high inter-individual variability was observed in the T group. Only 6 subjects out of 8, had values above 4, and the detection window last only from 2h to 8h after tablet intake. Even if the T/E was shown to be very specific (98%), the sensitivity of this parameter was only of 27%, leading to many false negative cases. The individual U03 had very low T/E values and, after genotyping, was found to be deleted in the gene coding for the enzyme UDP-glucuronosyltransferase 2B17 (UGT2B17), mainly responsible for the excretion of T as glucuronide-conjugated form in urine. The T/E of the individual U11 never reached the ratio 4, and he was considered as a fast excretor, whose excretion peak was missed at the selected time points. To highlight significant contributions of the 10 targeted analytes, a discriminant analysis was performed by building an N-PLS model with dependence on the placebo and T classes. While the sensitivity was higher than only based on the T/E ratio (62.5%) and an excellent specificity was maintained (88.9%), the detection window was not enhanced by using 10 analytes as compared to the T/E ratio.

Compounds	Concentrations [ng/mL]	Trueness	Repeatability	Intermediate Precision	LLOQ [ng/mL]	LOD [ng/mL]
TS	1	97%	28.4%	47.6%	2	1.0
	2	102%	17.1%	17.8%		
	10	96%	8.3%	13.1%		
	100	104%	11.4%	12.1%		
	200	109%	4.2%	8.9%		
ES	1	100%	17.8%	29.8%	4	1.0
	2	116%	21.4%	22.7%		
	10	101%	8.9%	10.5%		
	100	102%	3.6%	6.8%		
	200	92%	9.1%	10.7%		
DHEAS	50	97%	13.3%	13.1%	50	1.0
	500	102%	7.5%	7.6%		
	1,000	97%	8.5%	11.6%		
	3,000	93%	8.0%	12.2%		
	30,000/10	119%	17.8%	17.5%		
AS	50	108%	20.9%	21.6%	50	1.0
	500	106%	4.1%	7.7%		
	1,000	104%	9.8%	15.0%		
	3,000	93%	7.6%	13.1%		
	30,000/10	91%	5.9%	12.8%		
EtioS	50	108%	9.1%	10.2%	50	1.0
	500	93%	7.6%	11.6%		
	1,000	89%	5.0%	7.3%		
	3,000	99%	17.7%	18.3%		
	30,000/10	88%	4.5%	10.3%		

Table 1A: Validation parameters for the 10 investigated steroids

Compounds	Concentrations [ng/mL]	Trueness	Repeatability	Intermediate Precision	LLOQ [ng/mL]	LOD [ng/mL]
TG	5	85.8%	16.3%	18.9%	5	1.0
	10	100%	13.3%	14.1%		
	50	102%	10.6%	13.2%		
	100	99%	16.1%	15.9%		
	500	97%	3.8%	11.9%		
EG	5	93%	15.1%	16.3%	5	1.0
	10	104%	7.8%	13.8%		
	50	110%	4.1%	7.0%		
	100	108%	3.8%	8.4%		
	500	111%	10.7%	11.7%		
DHEAG	5	109%	14.7%	16.9%	5	1.0
	10	100%	9.0%	11.6%		
	50	106%	10.3%	11.8%		
	100	103%	7.6%	10.2%		
	500	97%	12.5%	14.4%		
AG	500	100%	10.1%	10.5%	500	5
	2,000	105%	5.4%	10.0%		
	4,000	101%	8.0%	10.6%		
	8,000	95%	7.6%	15.3%		
	80,000/10	98%	4.1%	4.6%		
EtioG	500	99.0%	17.5%	21.2%	500	5
	2,000	105%	4.8%	5.4%		
	4,000	100%	13.8%	13.4%		
	8,000	105%	11.9%	13.7%		
	80,000/10	95%	7.2%	10.5%		

Tabel 1B: Validation parameters for the 10 investigated steroids (continued)

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Sample	Group	Genotype	t00	t01	t02	t03	t04	t05
U 01	Placebo		0.46	0.78	0.74	0.52	0.93	0.60
U 06			1.54	0.59	1.11	0.40	0.61	0.62
U 09			0.57	0.57	0.68	0.47	0.56	0.52
U 10			0.88	0.81	0.44	0.86	1.15	0.76
U 13			0.10	-	0.08	0.11	0.10	0.05
U 18			0.36	1.17	0.39	0.45	0.66	0.92
U 20			0.67	0.39	0.42	0.94	0.65	0.92
U 24			3.42	2.06	1.74	2.10	2.76	2.46
U 25		del/del	0.01	0.05	0.14	0.06	0.09	0.04
U 03	Testosterone	del/del	0.17	0.05	0.09	0.05	0.08	0.14
U 05			0.87		15.32	1.41	0.61	0.78
U 07			1.00	2.77	29.01	16.67	2.05	0.67
U 11			0.32	0.36	0.94	0.50	0.29	0.40
U 14			4.84	3.10	23.02	68.75	20.59	3.16
U 17			0.60	0.63	13.21	0.98	0.98	0.61
U 19			0.24	0.35	5.81	8.85	0.98	0.41
U 27			0.78	0.61	4.44	10.96	1.27	0.76

Table 2: T/E ratios measured for each volunteer at time kinetic, with t00 = before tablet intake, t01= 0h, t02 = 4h, t03 = 8h, t04 = 24h and t05 = 200h after tablet intake

Extended Profile

The interest of an extended steroid profile was evaluated by using untargeted steroidomic analysis. UHPLC-QTOF-MS^E data were pre-processed and 5'750 peaks were detected automatically, corresponding to peak area at specific *m/z* and retention time. As the biological information was thought to be driven by the steroids themselves, a filter was applied to the data. This filter consisted in selecting all *m/z* corresponding to glucuronide-conjugated and/or sulfo-conjugated steroid, based on literature and biological knowledge (LipidMaps database [10]). After filtering, 234 steroid-like compounds were selected. A N-PLS model was built based on the 234 *m/z* and presented in **Figure 2A** and **B,** in the sample and time mode, respectively. The sample mode plot revealed a clear separation between the placebo and T classes. From the time mode graph, a trajectory could be highlighted by linking the time points chronologically. The trajectory presented a distinction between a basal state at t00 and t05, two intermediate situations (t01 and t04) and an acute phase of excretion (t02 and t03). By using 234 *m/z* variables, the detection window could thus be extended to 24h after 80 mg T undecanoate pill intake.

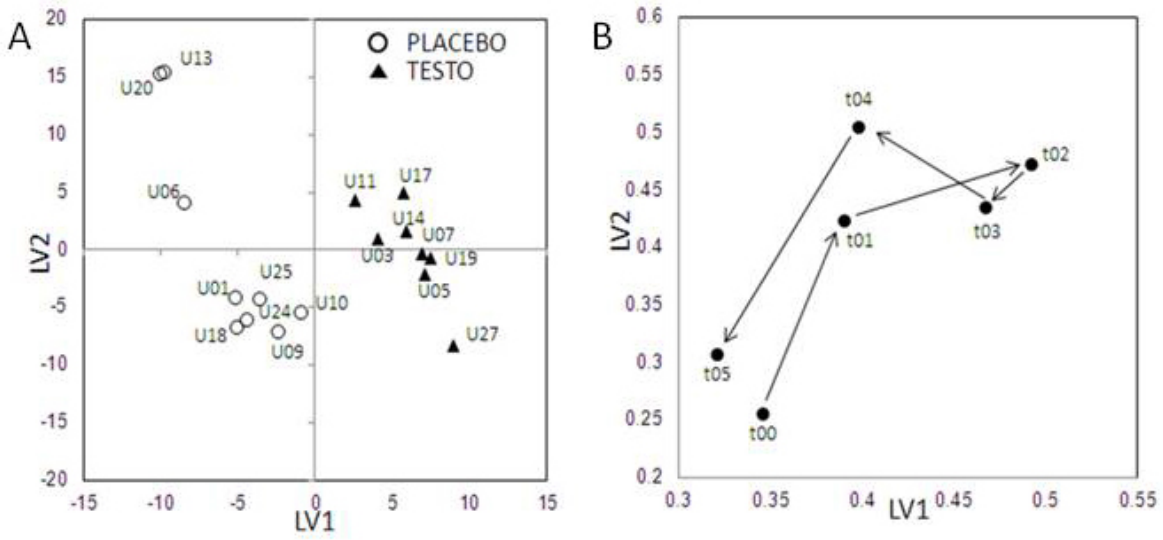

Figure 2: N-PLS-DA scatter plots for the extended steroid profile in the A) sample mode and B) time mode. Circles represented the individuals from the placebo group and triangles from the testosterone group

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Biomarkers Discovery

The information obtained with the previous model allowed highlighting 12 variables as potential biomarkers of T undecanoate intake. Sensitivity and specificity of each biomarker was evaluated by building receiver-operator characteristics (ROC) curves. Three highly predictive biomarkers were detected by this method. Retention time, *m/z* and fragments obtained in the second MS^E functions allowed to attribute potential structure of the analytes. Those biomarkers could be associated with an isomer of A-, Etio- or dihydrotestosterone (DHT)-sulfate, while another corresponded to hydroxylated form of A-, Etio- or DHT-glucuronide. The last marker was identified as DHT-glucuronide itself with comparison to a reference standard. This AAS is known to be a sensitive and selective marker of T undecanoate intake.

Conclusions

The steroidomic analysis presented in this study included a quantitative analysis of 10 targeted analytes from the steroid profile as intact glucuronide and sulfate conjugate, and could be applied to untargeted analysis. This platform allowed detecting known and novel markers of T undecanoate intake for anti-doping purpose. When using untargeted approach, the detection window could be extended to 24 h and three relevant biomarkers were identified. The detection of endogenous markers following testosterone undecanoate intake confirmed the interest of an untargeted profiling as a pertinent and valuable analytical strategy. Indeed, the phase II metabolism of steroids, including sulfate and glucuronide conjugate allowed for complementary information, in comparison with known biomarkers.

References

[1] WADA World Anti-Doping Agency. Reporting and evaluation guidance for testosterone, epitestosterone, T/E ratio and other endogenous steroids. Montreal (2004) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA_TD2004EAAS_Reporting_Evaluation_Testosterone_Epitestosterone_TE_Ratio_EN.pdf (access date 14.08.2012).
[2] Badoud, F., Grata, E., Boccard, J., Guillarme, D., Veuthey, J.-L., Rudaz, S. and Saugy, M. (2011). Quantification of glucuronidated and sulfated steroids in human urine by ultra-high pressure liquid chromatography quadrupole time-of-flight mass spectrometry. *Analytical and Bioanalytical Chemistry* **400,** 503-516.
[3] Plumb, R. S., Johnson, K. A., Rainville, P., Smith, B. W., Wilson, I. D., Castro-Perez, J. M. and Nicholson, J. K. (2006). UPLC/MSE; a new approach for generating molecular fragment information for biomarker structure elucidation. *Rapid Communications in Mass Spectrometry* **20,** 1989-1994.
[4] Boccard, J., Veuthey, J.-L. and Rudaz, S. (2010). Knowledge discovery in metabolomics: An overview of MS data handling. *Journal of Separation Science* **33,** 290-304.
[5] Baume, N., Schumacher, Y., Sottas, P.-E., Bagutti, C., Cauderay, M., Mangin, P. and Saugy, M. (2006). Effect of multiple oral doses of androgenic anabolic steroids on endurance performance and serum indices of physical stress in healthy male subjects. *European Journal of Applied Physiology* **98,** 329-340.
[6] Andersson, C. A. and Bro, R. (2000). The N-way Toolbox for MATLAB. *Chemometr. Intell. Lab.* **52** 1-4.
[7] Boccard, J., Badoud, F., Grata, E., Ouertani, S., Hanafi, M., Mazerolles, G., Lantéri, P., Veuthey, J.-L., Saugy, M. and Rudaz, S. (2011). A steroidomic approach for biomarkers discovery in doping control. *Forensic Science International* **213,** 85-94.
[8] WADA World Anti-Doping Agency. International Standard for Laboratories V7.0, Montreal (2012) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/ISL/WADA_Int_Standard_Laboratories_2012_EN.pdf (access date 14.08.2012).
[9] Food and Drug Administration, Guidance for Industry: Bioanalytical method validation, US Department of Health and Human Services, FDA, Centre for Drug Evaluation and Research, Rockville, MD (2001).
[10] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology, Q2 (R1), Geneva (2005).
[11] Fahy, E. and et al. (2009). Update of the LIPID MAPS comprehensive classification system for lipids. *J. Lipid Res.* **50,** S9-S14.