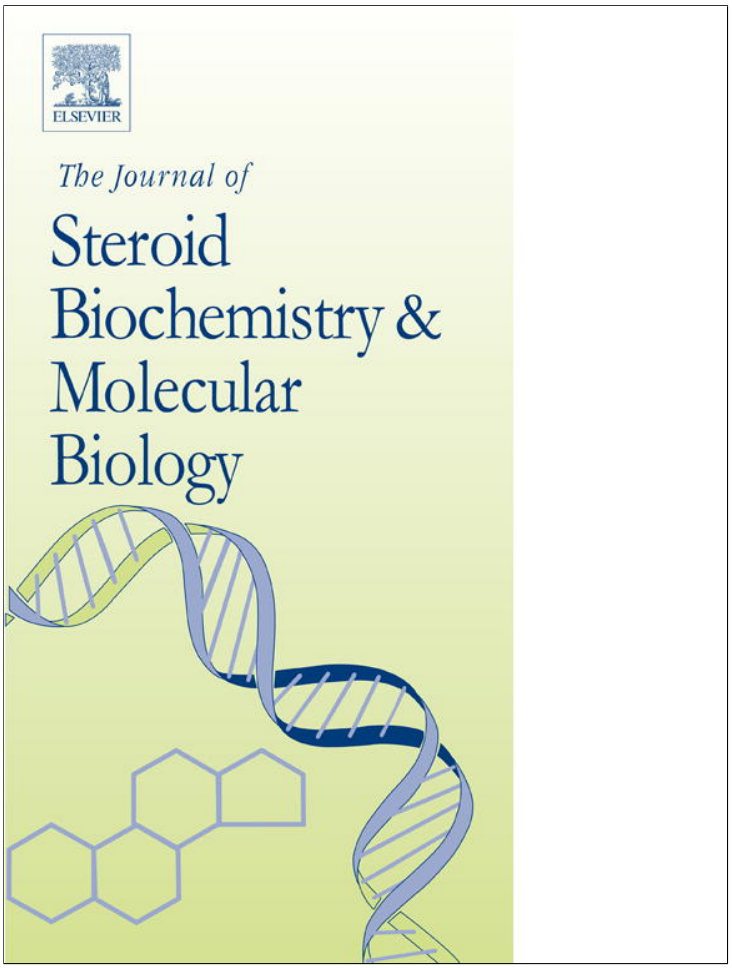

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Profiling of steroid metabolites after transdermal and oral administration of testosterone by ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

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ABSTRACT

The screening of testosterone (T) misuse for doping control is based on the urinary steroid profile, including T, its precursors and metabolites. Modifications of individual levels and ratio between those metabolites are indicators of T misuse. In the context of screening analysis, the most discriminant criterion known to date is based on the T glucuronide (TG) to epitestosterone glucuronide (EG) ratio (TG/EG). Following the World Anti-Doping Agency (WADA) recommendations, there is suspicion of T misuse when the ratio reaches 4 or beyond. While this marker remains very sensitive and specific, it suffers from large inter-individual variability, with important influence of enzyme polymorphisms. Moreover, use of low dose or topical administration forms makes the screening of endogenous steroids difficult while the detection window no longer suits the doping habit. As reference limits are estimated on the basis of population studies, which encompass inter-individual and inter-ethnic variability, new strategies including individual threshold monitoring and alternative biomarkers were proposed to detect T misuse.

The purpose of this study was to evaluate the potential of ultra-high pressure liquid chromatography (UHPLC) coupled with a new generation high resolution quadrupole time-of-flight mass spectrometer (QTOF-MS) to investigate the steroid metabolism after transdermal and oral T administration. An approach was developed to quantify 12 targeted urinary steroids as direct glucuro- and sulfo-conjugated metabolites, allowing the conservation of the phase II metabolism information, reflecting genetic and environmental influences. The UHPLC–QTOF-MS^E platform was applied to clinical study samples from 19 healthy male volunteers, having different genotypes for the UGT2B17 enzyme responsible for the glucuroconjugation of T. Based on reference population ranges, none of the traditional markers of T misuse could detect doping after topical administration of T, while the detection window was short after oral TU ingestion. The detection ability of the 12 targeted steroids was thus evaluated by using individual thresholds following both transdermal and oral administration. Other relevant biomarkers and minor metabolites were studied for complementary information to the steroid profile, including sulfoconjugated analytes and hydroxy forms of glucuroconjugated metabolites. While sulfoconjugated steroids may provide helpful screening information for individuals with homozygotous UGT2B17 deletion, hydroxy-glucuroconjugated analytes could enhance the detection window of oral T undecanoate (TU) doping.

1. Introduction

The screening of endogenous anabolic androgenic steroids (AAS) for doping control purpose is routinely implemented by assessing the steroid profile in urine. Monitoring ratios between precursors or biosynthetic metabolites of testosterone (T) helps to distinguish an exogenous intake from physiological levels. The most sensitive biomarker is the T glucuronide (TG) to epitestosterone glucuronide (EG) TG/EG ratio, which cut-off is set at 4 for suspicion of T or precursor administration [1]. Despite the high specificity of this parameter, the sensitivity of this test suffers from the short detection window and the high inter-individual variability due to natural elevated TG/EG ratios or genetic polymorphisms [2–5]. The majority of steroids are biotransformed into phase I and II metabolites

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Table 1
Study design.

Phases	Weeks	Day	Administration	Urine and blood samples collection
Control	1	Mo	–	C-t00 = 0 h; C-t01 = 2 h; C-t02 = 4 h; C-t03 = 8 h; C-t04 = 12 h
		Tu	–	C-t05 = 24 h
Patch	2	Mo	2 transdermal patches of 2.4 mg/24 h	P-t00 = 0 h; P-t01 = 2 h; P-t02 = 4 h; P-t03 = 8 h; P-t04 = 12 h
		Tu		P-t05 = 24 h
		We	2 transdermal patches of 2.4 mg/24 h	P-t06 = 48 h; P-t07 = 60 h {All spot urine samples produced during 48 h
		Th		P-t08 = 72 h
		Fr		P-t09 = 96 h
Wash out	3	Mo		t10 = 118 h
	4	Mo		t11 = 336 h
Oral	5	Mo	2 oral pills of 40 mg	O-t00 = 0 h; O-t01 = 2 h; O-t02 = 4 h; O-t03 = 8 h; O-t04 = 12 h
		Tu		O-t05 = 24 h
		We	2 oral pills of 40 mg	O-t06 = 48 h; O-t07 = 60 h {All spot urine samples produced during 48 h
		Th		O-t08 = 72 h
		Fr		O-t09 = 96 h

(glucuro- and sulfo-conjugates) to facilitate their excretion in urine. Among the UDP-glucuronyl transferases (UGT), the UGT2B17 is the most important isoform involved in T glucuronidation and is highly subjected to polymorphism. It was indeed reported that 40% of the individuals having a del/del genotype for the *UGT2B17* gene coding for the UGT2B17 enzyme, a polymorphism representing two third of the population of Asian ethnic origin, did not show any TG/EG elevation after T administration [6]. Moreover, today's doping habits consist in low-doses and topical route of administration, according to testimony of top-level athletes, to avoid peak concentration excretion [7]. Then, the current TG/EG ratio threshold and other steroid profile parameters might not be sufficiently sensitive to detect low doses and transdermal T route of administration [8,9]. To avoid this limitation, subject-based monitoring approach was proposed by setting individual basal threshold levels instead of population reference limit as suitable tool for steroid profiling, as proposed for the future Athlete Steroidal Passport (ASP) [10–12].

With the perspective of using multiparametric indicators to reinforce the steroid profile detection ability, the research of novel biomarkers or minor metabolites is expanding to improve current detection windows of AAS doping [5,13–15]. To date, gas chromatography (GC) coupled to mass spectrometry (MS) or tandem MS (MS/MS) remains the gold standard for steroid analysis, but necessitates hydrolysis and derivatization prior to the analysis, leading to loss of phase II metabolism information. Besides, several liquid chromatography (LC) linked to MS(/MS) developments led to the direct detection of glucuro- and sulfo-conjugated metabolites as well as alternative markers released after alkaline treatment [5,16–21].

In this study, a new generation quadrupole time-of-flight mass spectrometer coupled to ultra-high pressure liquid chromatography (UHPLC–QTOF-MS) was evaluated for its ability to assess the steroid metabolism after T administration. The procedure was adapted from a method previously reported as a relevant strategy for the quantification of AAS in urine as direct glucuro- and sulfo-conjugated metabolites [17] and applied to real-case samples issued from a clinical trial. The former method was developed with a previous generation QTOF mass analyzer allowing good mass accuracy (2–5 ppm) and resolution (12,000 FWHM), while sensitivity and dynamic range of the instrument could be further improved. Meanwhile, a new generation QTOF instrument was commercially available, promising excellent performance, particularly in terms of resolution, sensitivity, speed and dynamic range for quantitative analysis. The performance of the new generation instrument was compared with the previous approach [17], to evaluate its benefits to investigate the steroid metabolism. For that purpose, urine samples from a clinical trial protocol, collected after T transdermal and T undecanoate (TU) administration to 19 healthy male volunteers, having different UGT2B17 genotypes (del/del, ins/del and ins/ins), were analyzed. The variation of twelve targeted steroid metabolites quantified in this study in more than 500 urine samples was evaluated with respect to both route of T administration based on individual threshold levels.

2. Experimental

2.1. Study design

The cohort recruited for the clinical trial included 19 healthy young men, aged 19–28 (mean 24.3 ± 2.7 years) with a body mass index (BMI) comprised between 18.3 and 27.2 (mean 23.1 ± 2.4 kg/m^2). 4 volunteers were rejected to join the cohort, as they did not fulfill the inclusion criteria, while 2 subjects dropped out for personal reason. A complete medical history and physical exam was performed at inclusion in the study. Exclusion criteria were: regular drug, alcohol or tobacco use, anabolic or ergogenic substance intake, dyslipidemia, hypercholesterolemia, androgen-dependent tumor, hyperprolactinemia, endocrine or metabolic disturbance, use of thyroid hormone or anti-thyroid agent, cardiac, renal or hepatic deficiency. In addition, the volunteers did not practice sport at a competitive level, did not take any medication and refrained from ingesting alcohol during the study. All subjects gave signed consent form and the protocol was authorized by the Ethical Commission for the Clinical Research of the Faculty of Biology and Medicine (University of Lausanne, Lausanne, Switzerland) and Swissmedic (Protocol n°155/11). This study was conducted at the Clinical Research Center of the CHUV/UNIL. The participants received T as presented in Table 1. Control samples (C) were gathered during the first week, mainly on Monday. Kinetics urine and blood spots were collected at time C-t00 = 0 h, C-t01 = 2 h, C-t02 = 4 h, C-t03 = 8 h, C-t04 = 12 h and C-t05 = 24 h. During the second week, they received twice 2 T transdermal systems of 2.4 mg/24 h (Testopatch®, Pierre Fabre Pharma GMBH, Freiburg, Germany) on Monday and Wednesday. The patch system (P) was stuck on the shoulder and removed after 48 h. During that time urine and blood were taken at times P-t00 = 0 h, P-t01 = 2 h, P-t02 = 4 h, P-t03 = 8 h, P-t04 = 12 h, P-t05 = 24 h and P-t06 = 48 h. Time points P-t07 = 60 h, P-t08 = 72 h and P-t09 = 96 h were collected after the application of the second patch. Apart from the mentioned collection times, all the urines produced were gathered during 48 h after the application of the first patch. During the two following weeks, the first morning urine as well as a blood sample was collected on Mondays. Following a wash-out period of 2 weeks, 2 TU tablets of 40 mg (Andriol Testocaps®, Essex Chemie AG, Luzern, Switzerland) were taken orally by the volunteers on Monday and Wednesday. During the fifth week, urine and blood specimens were

collected after oral pill ingestion (O) at the same time points to those of T patch administration (O-t01-09). Blood tests included 1 EDTA tube (potassium EDTA) of 7 mL and 2 serum tubes (clotting activator) of 4.5 mL. EDTA tubes were directly stored at +4 °C before analysis, while serum samples were centrifuged at 1500 × *g* for 15 min after 15 min stabilization at room temperature. Serum aliquots were kept frozen at −20 °C until analyses. Urine samples were divided into 20-mL vials and frozen at −20 °C before analysis within 6 months. In the present paper, blood samples were only used for genotyping.

2.2. Genotyping of UGT2B17 and UGT2B15

Genomic DNA was extracted from blood samples and prepared using Qiamp DNA Blood Mini Kit (Hilden, Germany). PCR included genomic DNA, Taqman SNP Genotyping Assays kit and 2× Taqman universal master mix and were run on a API 7500 Fast (Applied Biosystems, Forset City, CA, USA). UGT2B17 and UGT2B15 genotyping was conducted on blood samples from the 19 volunteers. Genotypes were well balanced with 5 del/del, 7 ins/del and 7 ins/ins genotypes for UGT2B17 and 5 YY, 4 DD and 10 YD for the rs1902023 SNP of UGT2B15.

2.3. UHPLC–QTOF-MS analyses

2.3.1. Chemicals and reagents

The following commercially available standards of AAS in glucuro- and sulfo-conjugated form were acquired: 4-androsten-17β-ol-3-one sulfate sodium salt (testosterone sulfate, TS), 4-androsten-17α-ol-3-one sulfate sodium salt (epitestosterone sulfate, ES), 5β-androstan-3α-ol-17-one sulfate sodium salt (etiocholanolone sulfate, EtioS), 5-androsten-3β-ol-17-one sulfate sodium salt (dehydroepiandrosterone sulfate, DHEAS), 4-androsten-17β-ol-3-one glucosiduronate (testosterone glucuronide, TG) 4-androsten-17α-ol-3-one glucosiduronate (epitestosterone glucuronide, EG), 5β-androstan-3α-ol-17-one glucosiduronate (etiocholanolone glucuronide, EtioG), 5-androsten-3β-ol-17-one glucosiduronate (dehydroepiandrosterone glucuronide, DHEAG), 5α-androstan-17β-ol-3-one glucosiduronate (dihydrotestosterone glucuronide, DHTG) and 5α-androstan-3β, 17β-diol-3-glucosiduronate (α-diol-3-glucuronide, 5αββ-AdiolG) were purchased from Steraloids (Newport, RI, USA). 5α-androstan-3α-ol-17-one sulfate triethylammonium salt (androsterone sulfate, AS), 5α-androstan-3α-ol-17-one-3α-D-glucuronide (androsterone glucuronide, AG), [16,16,17α-$^{2}H_3$]androst-4-en-17α-ol-3-one sulfate triethylammonium salt (TS-d_3), [16,16,17β-$^{2}H_3$]androst-4-en-17β-ol-3-one sulfate triethylammonium salt (ES-d_3), [2,2,3β,4,4-$^{2}H_5$]5β-androstan-3α-ol-17-one sulfate triethylammonium salt (EtioS-d_5), [2,2,4,4-$^{2}H_4$]5α-androstan-3α-ol-17-one sulfate triethylammonium salt (AS-d_4), [16,16,17α-$^{2}H_3$]androst-4-en-17β-ol-3-one glucuronide (TG-d_3), [16,16,17α-$^{2}H_3$]androst-4-en-17α-ol-3-one glucuronide (EG-d_3), [16,16,17β-$^{2}H_3$]5α-androstan-17β-ol-3-one glucosiduronate (DHTG-d_3) [2,2,4,4-$^{2}H_4$]5α-androstan-3α-ol-17-one glucuronide (AG-d_4) were purchased from the Australian Government National Measurement Institute (Pymble, Australia). Ultra-pure water was provided by a Milli-Q system from Millipore (Bedford, MA, USA) and ULC–MS quality of water and acetonitrile (CH_3CN) were obtained from Biosolve (Chemie Brunschwig, Basel, Switzerland), while formic acid (FA) and ammonium hydroxide (NH_4OH) were supplied from Fluka (Buchs, Switzerland) and methanol (CH_3OH) by Merck (Darmstadt, Germany). All stock standards solutions were prepared in CH_3OH at a concentration of 1 mg/mL. Stock internal standard (I.S.) solutions were obtained in CH_3OH at a concentration of 100 μg/mL and at 1 mg/mL for AG-d_4. Working I.S. solution was obtained by spiking 100 μL of TS-d_3 and ES-d_3 stock solutions, 200 μL of TG-d_3, EG-d_3, DHTG-d_3 and AG-d_4, 500 μL of AS-d_4 and Etios-d_5 to 10 mL of CH_3OH. All of the solutions were stored at −20 °C in glass tubes fitted with PTFE caps.

2.3.2. Sample preparation

The sample preparation was adapted from a previous work [17] and consisted in the solid phase extraction (SPE) on Oasis HLB (Waters, Milford, MA, USA) cartridges 30 mg (30 μm particle size) in the 96-well plate format to extract selectively glucuro- and sulfo-conjugated AAS from urine samples. Briefly, the protocol was as follows: conditioning was performed with 500 μL of CH_3OH and equilibration with 500 μL of an aqueous solution containing 2% FA. 1 mL of urine with 1 mL of 2% FA solution spiked with 10 μL of I.S. working solution was loaded in the wells followed by a first wash with 1 mL of a solution of 2% FA and a second with 1 mL of a solution of NH_4OH 5%/CH_3OH (90%/10%, v/v). The elution step consisted of 500 μL of 95% CH_3OH/5% water. Eluates were evaporated to dryness under a gentle air stream and dry residue was reconstituted with 100 μL of a mixture of 30% CH_3CN/70% water and injected in the LC–MS system.

2.3.3. UHPLC–MS conditions

An Acquity UPLC system (Waters) equipped with Acquity UPLC columns (BEH C_{18} 150 mm × 2.1 mm, 1.7 μm) and a Van Guard (Waters) pre-column (BEH C_{18} 5 mm × 2.1 mm, 1.7 μm) were used for the chromatographic separation. The column was maintained at 25 °C and the flow rate at 300 μL/min. The mobile phase was (A) 0.1% FA in water, and (B) 0.1% FA in CH_3CN. The gradient started linearly from 5% to 37% B over 25 min, followed by an increase to 95% B for 3 min, the system was then re-equilibrated for 8 min. The injection volume was fixed at 10 μL in the full loop mode and samples were maintained at 4 °C in the autosampler.

MS analyses were performed using a Synapt G2 HDMS mass spectrometer (quadruople time-of-flight, QTOF, Waters) equipped with an Intellistart fluidic system and an ESI source operated in the negative mode. The following parameters were set on the MS: capillary voltage at 2.4 kV, sampling cone at 50 V, extraction cone at 5 V, source temperature at 120 °C, desolvation temperature 360 °C, cone gas flow at 20 L/h and desolvation gas flow at 850 L/h. A solution of leucine-enkephalin at 2 ng/mL (Sigma–Aldrich, Buchs, Switzerland) was infused through the Lock Spray probe at 10 μL/min with a scan time of 0.02 s and an interval of 15 s. The MS^E mode was selected to acquire the data. Two functions were operated simultaneously, a first function at low collision energy (6 eV) over the mass range 100–600 *m/z* and a second function with a collision energy ramp (35–50 eV) over the *m/z* 50–600. Data were collected in the resolution mode operating in V-optics centroid with a scan time of 0.2 s and an interscan delay of 0.02 s.

Data acquisition, data handling and instrument control were performed using MassLynx Software (Waters). TargetLynx Software (Waters) was used to calibrate, quantify and process the data and MarkerLynx XS Software (Waters) was used to automatically extract the raw data. Receiver-Operating Characteristics (ROC) curves analysis was performed in XLSTAT (Addinsoft).

2.3.4. Validation and quantitative analysis

For comparison with the original method carried out in [17], the same validation protocol was applied to the new generation QTOF data. Briefly, 3 consecutive series were performed with calibration standards at 4 levels ($k = 4$) and validation standards at 5 levels ($k = 5$), each of them in triplicate ($n = 3$). Calibration curves were built based on the peak area ratio of 5αββ-AdiolG, TG and DHEAG to TG-d_3, EG to EG-d_3, AG and EtioG to AG-d_4, DHTG to DHTG-d_3, TS to TS-d_3, ES and DHEAS to ES-d_3, AS to AS-d_4 and EtioS to EtioS-d_5. The curves were generated over the range from 1

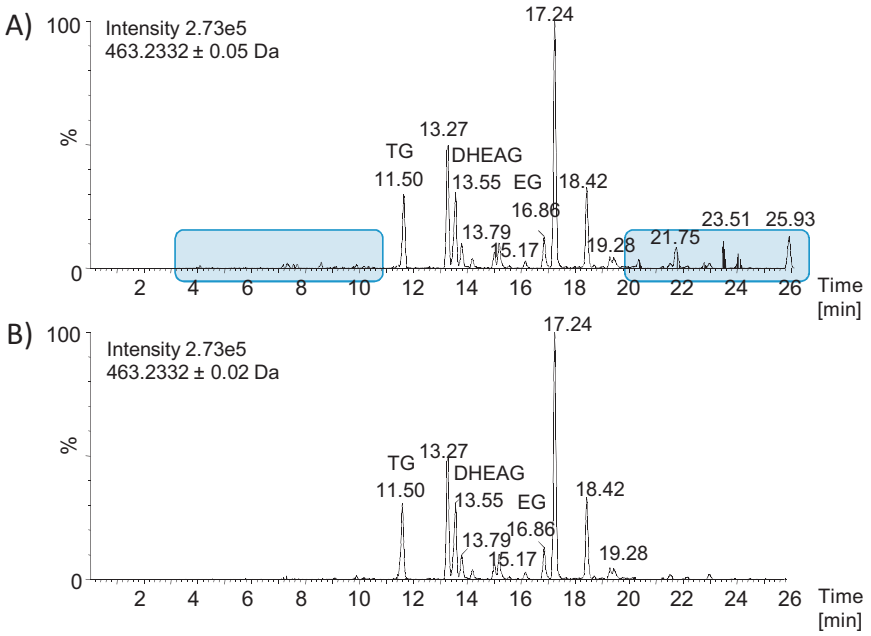

Fig. 1. Extracted ion chromatogram from a morning urine sample of volunteer 11. A mass window of 0.05 Da (A) and 0.02 Da (B) was selected to extract the trace 463.2332 corresponding to TG, EG and DHEAG.

to 200 ng/mL for TS and ES, 5 to 500 ng/mL for TG, EG and DHEAG, 2 to 100 ng/mL for 5αββ-AdiolG, 2 to 500 ng/mL for DHTG, 50 to 3000 ng/mL for DHEAS, AS and EtioS, and 500 to 8000 ng/mL for AG and EtioG, according to previous studies on transdermal and oral administration of T and instrument sensitivity [7–9,22,23].

Results were expressed in terms of trueness (relative bias), repeatability (relative standard deviation (RSD) of averaged intra-day standard deviation) and intermediate precision (RSD of inter-day standard deviation). Urine samples from the clinical trial were defrosted at ambient temperature and randomized before analysis. An external standard calibration with four levels of concentration was used in duplicate with a 1/*x*-weighting correction to quantify the metabolites in real-case urine samples.

Matrix effects were evaluated by comparing peak areas of analytes spiked in extracted blank urines (pooled urines from one boy and two girls less than 3 years old with negligible amount of all steroids) to peak areas of the corresponding reference standard prepared in water, according to the procedure described in Badoud et al. [17]. Quality control (QC) samples were added at the beginning and at the end of each analytical batch and all the samples were extracted in duplicate. The batch was rejected if the QC nominal value was not comprised between the intervals of ±15%, as recommended by the Food and Drug Administration [24].

2.4. GC–MS analyses

The GC–MS analyses were performed according to the accredited procedure for the quantification of the steroid profile in urine, including hydrolysis of glucuroconjugated part and derivatization prior to injection [25].

3. Results and discussion

The purpose of this study was first to evaluate the performance of a new generation QTOF mass spectrometer for the targeted quantitative analysis of 12 endogenous anabolic steroids measured as glucuro- and sulfo-conjugated metabolites in urine samples. Secondly the method was applied to samples collected during a clinical trial to investigate the metabolism of T after administration by topical and oral route. The sensitivity and accuracy of each biomarker with respect to the study cohort was evaluated via ROC curves analysis to compare the response of the 12 markers via the 2 routes of administration. Then individual thresholds for each volunteer were set up for all measured biomarkers to assess their ability to detect T doping and evaluate their usefulness by using an approach similar to that used for the forthcoming ASP.

3.1. Comparison between two generations of QTOF mass spectrometers

QTOF-MS linked to UHPLC is a platform of choice for metabolite profiling, allowing fast and sensitive methods. However, these mass analyzers were generally found to have poor quantitative performance with higher intra- and inter-day variability and lower dynamic range [26]. Recently, the quantitative capabilities of QTOF were improved with the development of new generation instruments. In the present study, the qualitative and quantitative capabilities of the new technologies were evaluated with respect to a former method developed on a QTOF mass spectrometer from older generation, i.e., QTOF Premier [17]. Main modifications on the new generation QTOF concerned enhancement in resolution, reaching 40,000 FWHM, and mass accuracy (1 ppm). Moreover, the dynamic range was improved to 5 orders of magnitude, without using dynamic range enhancement algorithm.

The validation protocol conducted on the first generation instrument was carried out on the new instrument system to evaluate the accuracy and precision of this new generation mass spectrometer on 12 targeted conjugated steroids. The trueness, repeatability and intermediate precision were assessed for both mass spectrometers by using validation standards corresponding to spiked blank urine (a urine obtained after pooling urines from one boy and two girls less than 3 years old with negligible amount of all steroids) at 4 concentration levels in triplicate (Table 2). It is noteworthy that DHTG was not included in the previous protocol, but was added in the present procedure. The validation parameters presented allowed therefore the use of the method for the quantitative analysis of this compound. While the trueness was in the same range for both mass spectrometers (i.e., 85–115.5% and 85.8–116%),

Table 2
Validation parameters for Synapt G2 and QTOF Premier mass spectrometers. Numbers highlighted in bold indicate the best values obtained for the new generation system.

Compounds	Concentrations (ng/mL)	Trueness		CVr		CVR		LLOQ		LOD	
		SYNAPT G2 (%)	QTOF Premier (%)	SYNAPT G2 (%)	QTOF Premier (%)	SYNAPT G2 (%)	QTOF Premier (%)	SYNAPT G2	QTOF Premier	SYNAPT G2	QTOF Premier
TG	5	105.8	85.8	**5.3**	16.3	**10.1**	18.9	5	5	**0.5**	1
	50	108.4	102.0	**2.1**	10.6	**3.7**	13.2				
	100	100.3	99.0	**1.7**	16.1	**10.9**	15.9				
	500	106.8	97.0	4.1	3.8	**6.6**	11.9				
EG	5	104.1	93.0	**10.3**	15.1	**15.2**	16.3	5	5	**0.5**	1
	50	105.9	110.0	5.5	4.1	11.7	7.0				
	100	100.7	108.0	**2.6**	3.8	**4.9**	8.4				
	500	90.7	111.0	**5.9**	10.7	**11.5**	11.7				
DHEAG	5	96.8	109.0	**12.5**	14.7	**9.5**	16.9	5	5	**0.5**	1
	50	90.4	106.0	**3.7**	10.3	**9.6**	11.8				
	100	94.7	103.0	**4.6**	7.6	**5.3**	10.2				
	500	100.5	97.0	**6.1**	12.5	**5.3**	14.4				
AG	500	90.2	100.0	**7.5**	10.1	11.3	10.5	500	500	**1**	5
	2000	99.5	105.0	6.7	5.4	14.5	10.0				
	4000	103.8	101.0	**1.3**	8.0	**1.4**	10.6				
	8000	95.1	95.0	**7.4**	7.6	**7.9**	15.3				
EtioG	500	95.2	99.0	**5.3**	17.5	**12.1**	21.2	500	500	**1**	5
	2000	104.1	105.0	11.1	4.8	8.4	5.4				
	4000	94.7	100.0	**5.7**	13.8	**9.0**	13.4				
	8000	99.9	105.0	14.2	11.9	**11.2**	13.7				
αββ-AdiolG	2	107.7	–	**4.1**	–	**5.4**	–	**2**	5	**0.5**	1
	5	100.5	102.0	**7.2**	9.9	15.8	8.5				
	50	96.1	96.0	7.2	6.3	12.1	7.3				
	100	88.2	110.0	**3.2**	8.4	8.6	8.0				
DHTG	2	93.5	–	2.4	–	6.0	–	2	–	0.5	–
	10	115.5	–	1.6	–	6.5	–				
	200	102.0	–	3.4	–	11.2	–				
	500	99.0	–	1.6	–	12.4	–				
TS	1	113.0	97.0	**1.9**	28.4	**2.8**	47.6	**1**	2	**0.5**	1
	2	85.1	102.0	**2.0**	17.1	**5.4**	17.8				
	50	102.5	–	11.0	–	9.9	–				
	200	99.8	109.0	**3.5**	4.2	14.9	8.9				
ES	1	99.1	100.0	**10.8**	17.8	**15.6**	29.8	**1**	4	**0.5**	1
	2	102.5	116.0	**8.3**	21.4	**9.2**	22.7				
	50	94.9	–	2.0	–	3.1	–				
	200	86.2	92.0	**2.2**	9.1	**2.4**	10.7				
DHEAS	50	90.7	97.0	**5.6**	13.3	**6.7**	13.1	50	50	**1**	0.5
	500	110.4	102.0	**6.7**	7.5	9.7	7.6				
	1000	98.7	97.0	**4.0**	8.5	**6.8**	11.6				
	3000	89.9	93.0	**1.3**	8.0	**4.4**	12.2				
AS	50	85.0	108.0	**4.1**	20.9	**7.8**	21.6	50	50	**0.5**	1
	500	101.0	106.0	7.0	4.1	10.5	7.7				
	1000	92.2	104.0	**9.0**	9.8	**11.1**	15.0				
	3000	94.3	93.0	**5.4**	7.6	**4.9**	13.1				
EtioS	50	98.8	108.0	**2.0**	9.1	13.9	10.2	50	50	**0.5**	1
	500	106.5	93.0	**7.0**	7.6	**10.0**	11.6				
	1000	107.7	89.0	**3.5**	5.0	12.4	7.3				
	3000	108.4	99.0	**12.2**	17.7	**9.9**	18.3				

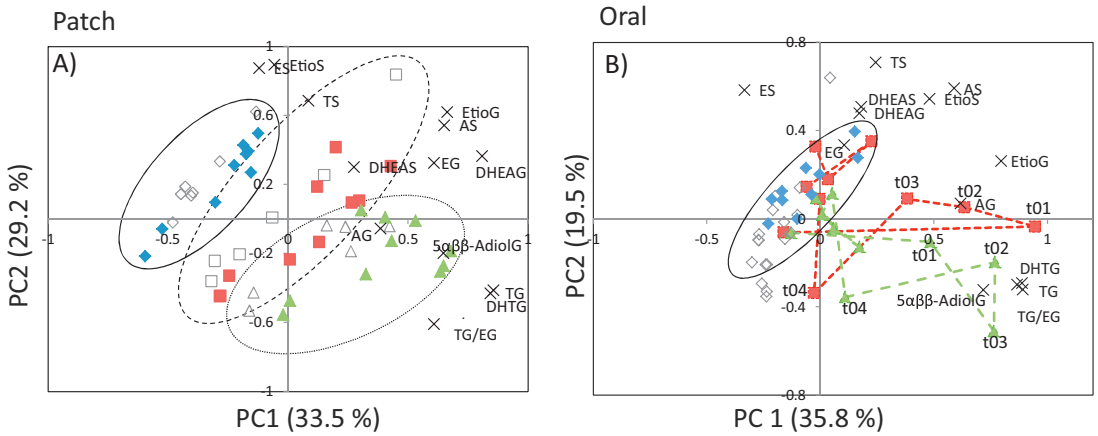

Fig. 2. Principal component analysis (PCA) biplot combining samples distribution (scores) and analytes' contributions (loadings) for the 12 targeted steroids. (A) Transdermal T patch administration and (B) oral TU pills intake. Empty ◊, □ and Δ correspond to the control samples from the del/del, ins/del and ins/ins UGT2B17 genotype, respectively. Full ⧫, ■ and ▲ are related to samples exposed to transdermal or oral T, for the del/del, ins/del and ins/ins UGT2B17 genotype, respectively. X represents the contributions of the targeted steroids. The variables were represented by PC1 and PC2 on the biplot.

the intra-day and inter-day variability was drastically lower on the new generation instrument. Indeed, the repeatability observed on the previous QTOF was between 3.8% and 28.4%, and was reduced to 1.3–14.2% on the new instrument. The same observation was done with the intermediate precision, lowered from the range 5.4–47.6% to 1.4–15.8%, respectively. Better precision was obtained with the new generation system, especially at low concentrations and the lower limits of quantification (LLOQ) could be lowered for 5αββ-AdiolG, TS and ES. Moreover, the excellent repeatability allowed the calibration curve to be run in duplicate instead of triplicate, and a diminution of the analysis time of around 2 h per batch.

Thanks to the high resolution and mass accuracy on the new generation instrument, ion chromatograms could be extracted with a mass window of 0.02 Da, instead of 0.05 Da. The extracted ion chromatogram presented in Fig. 1A and B, corresponded to a morning control urine sample analyzed on the new generation instrument for volunteer 11. The selectivity was better when extracting the ions with a narrower mass window, due to noise reduction, while no loss in sensitivity was observed.

The measured matrix effects were in the same range (80–111%) as reported with the previous method, since the sample preparation and UHPLC separation were similar.

The quantitative measurements performed for the 12 targeted analytes, expressed as mean and median concentration over a 24 h period in the control, patch and oral groups can be found in Supplementary Table 1. From the broad range of concentrations and urine specific gravity, most of the samples had concentrations of endogenous steroids in the calibration range. For less than 3% of the samples, no peaks were detected for DHEAG, EG, AG, EtioG, TS, ES, DHEAS, AS and EtioS; around 10% of the samples were below the LOD for TG; while 20% and 77% of the samples were under the LOD for DHTG and 5αββ-AdiolG, mostly among the del/del genotype. The TG/EG ratios were thus estimated from the GC–MS measurement of T for each sample with TG found under the LOD by LC–MS (see Supplementary Table 1). Finally, around 15% of the samples had values under the LLOQ of the method for TG, EtioG, TS and DHEAS, while less than 10% of the samples ranged between LOD and LLOQ for the other analytes. Highly concentrate samples were diluted to fit the calibration range, and this concerned around 5% of the samples predominantly for AG, EtioG, AS, EtioS, as well as for TG after oral TU ingestion and about 15% of the DHEAS measurement.

Eventually, to ensure the accuracy of the quantitative measurement, the 532 urine samples collected during the clinical trial were analyzed in parallel by the GC–MS WADA accredited method. The concentrations of the glucuroconjugated analytes that were measured with both procedures (i.e., TG, DHEAG, EG, DHTG, AG and EtioG) were thus compared. Bland–Altman analyses and correlation curves (see Supplementary Fig. 1) reveal a good correlation between the methods. The determination coefficient were higher than 0.77, except for DHEAG, whose determination coefficient was 0.56. Indeed, for the quantification of this analyte no isotope-labeled internal standard was used to correct for matrix effect. Therefore, a difference in selectivity could explain the variability in measurement from the LC–MS in comparison with the GC–MS method. Moreover, even if the correlation between both methods was good for EG, a systematic overestimation of the concentration could be observed from the correlation curve. This could be attributed to potential contributions of co-eluting isomers present in young male urines in comparison to the blank urine matrix used for the calibration curve, which consisted in a pooled urine from one boy and 2 girls of less then 3 years old with negligible amount of steroids [17]. Additionally, the measurement of DHTG was underestimated in comparison with the GC–MS procedure, which could be explained by the fact that most of the measurements were near the LLOQ of the two methods, as stated previously. However, as the concentrations of the compounds were in the same order of magnitude for both procedures, the method presented in this study was considered suitable for the quantification of the glucuroconjugated steroids. Assessing the fitness of the two methods on the basis of more than 500 values drastically increased the confidence in the accuracy of the measurements. The UHPLC–QTOF-MS^E platform offered good quantitative performance with direct determination of glucuro- and sulfo-conjugated steroids. The validation and comparison with an accredited method thus revealed that the quantitative performance of the method was fit for the purpose of the study.

3.2. Exploratory analysis of the dataset

Urine samples collected from the 19 healthy male volunteers during the clinical trial were randomized and analyzed in duplicate following the method described in this paper. The cross-over design of the clinical trial was used to reduce the variability and increase the confidence of the results by using each volunteer as its own control. Moreover, genotype and more particularly UGT2B17 polymorphism, reported to explain 66% of the inter-individual steroid excretion variability [6], was considered as an inclusion criteria. UGT2B17 and UGT2B15 polymorphisms were equally distributed among the volunteers. UGT2B15 which is also involved in the

glucuroconjugation of endogenous anabolic steroid, such as T and DHT, was also genotyped for the D85Y polymorphism. However, as this polymorphism is not related to the deletion of the enzyme, a weak impact on the glucuroconjugation of the steroids was reported [27].

Twelve endogenous steroids were monitored in each sample and the effect of urine dilution on their concentration was adjusted with the specific gravity by using the following relation:

$$C_{1.020} = \frac{1.020 - 1}{\text{Specific gravity sample} - 1} \times C_{\text{sample}}$$

The specific gravity of the samples ranged from 1.004 to 1.035. The data collection points were selected according to previous pharmacokinetic studies on T oral and transdermal application, revealing acute increase in concentration between 2 h and 12 h after oral TU ingestion [3,5,17,28], while the concentration of T was shown to rise slightly after patch administration during the 9 first hours [7,8].

A summary data table of 532 urine samples × 12 steroid conjugates (TG, EG, DHEAG, αββ-AdiolG, DHTG, AG, EtioG, TS, ES, DHEAS, AS and EtioS) was obtained. As a first step of data analysis, two principal component analysis (PCA) models were calculated separately to assess the trends related to both T administration routes compared to control urines. PCA biplots are presented in Fig. 2A and B for the analysis of control samples (C-t00–05) and transdermal T administration (P-t00–09) or oral pill intake (O-t00–09), respectively. As samples collected at different time points constituted homogeneous clusters, no chronological trend could be highlighted on the PCA biplot of the model including control and transdermal data (Fig. 2A). Therefore, T patch administration could not reveal a detectable influence on the steroid profile suggesting that the induction of the phenomenon was too small. The effect of the treatment was confounded with the biological variability and could certainly be attributed to the low dose and bioavailability as well as the slow kinetic release of topical system. Nevertheless, three clusters could be clearly distinguished on the biplot, corresponding to the three UGT2B17 genotypes and indicating a gradation of steroid levels (del/del > ins/del > ins/ins). This observation suggests that the basal level of the steroid profile is closely related to the UGT2B17 genotype and correlates with the TG/EG level in urine [29]. A marked dependence of the androgens was observed with respect to the UGT2B17 genotypes, as the main metabolites driving the separation highlighted from the loadings, i.e., variables contributions were TG/EG, TG and DHTG. As those specific metabolites are major substrates of UGT2B17, they underlined the basal enzyme activity and corroborated the hypothesis of a strong dependence of the TG/EG variability on UGT2B17 polymorphisms [6].

The PCA biplot of samples from volunteers exposed to 80 mg oral TU and controls (Fig. 2B) revealed a different picture. While no effect of this high dose could be highlighted for the del/del UGT2B17 genotype, a clear trajectory was revealed for the inserted heterozygotous and homozygotous individuals by linking the time points. Del/del individuals remained apparently unaffected by the treatment, as the samples were clustered with controls, but time points corresponding to O-t01, O-t02, O-t03 and O-t04 (2, 4, 8 and 12 h post-dose) of ins/del and ins/ins genotypes were clearly separated from the control samples following a chronological trajectory. By these means, an acute excretion state, starting from 2 h to 12 h after TU pill intake could be distinguished from a basal level, and the analysis of the variables' contributions indicated TG/EG, TG, DHTG and 5αββ-AdiolG as compounds driving the trend highlighted after TU pill administration.

Among the 12 targeted glucuro- or sulfo-conjugated analytes, glucuroconjugated metabolites (TG/EG, TG and DHTG) were the best indicators of oral TU consumptions. Nevertheless, high doses (80 mg) of TU were necessary to modify the steroid profile and the alterations could be only detected between time points O-t01 and O-t04, corresponding to 2–12 h after intake.

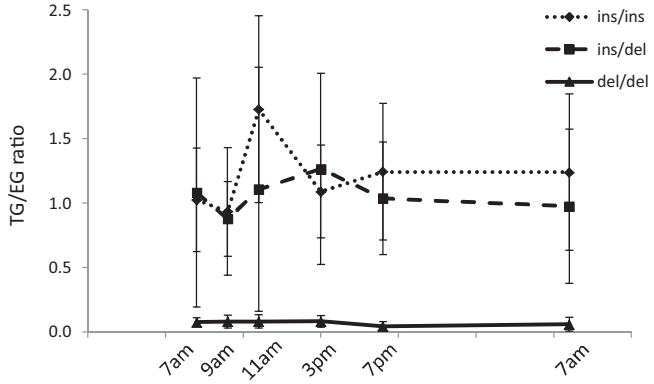

Fig. 3. Mean basal TG/EG ratio follow-up expressed with confidence interval at 95% ($CI_{95\%}$). Dotted line corresponded to individuals with ins/ins genotype ($n = 7$), dashed line to ins/del genotype ($n = 7$) and full line to the del/del genotype ($n = 5$).

3.2.1. Steroid profile in control samples

As T follows a circadian rhythm of secretion, the daily intra-individual variability of the steroid profile levels was evaluated by collecting samples at 6 time points during the first week of the clinical trial. Spot urine samples were collected at 7 am, 9 am, 11 am, 3 pm, 7 pm and 7 am in the following morning. The 12 targeted steroids were quantified to estimate baseline levels in the control group. The averaged TG/EG is presented in Fig. 3 for each genotype and the diurnal mean, expressed with the confidence interval at 95% ($CI_{95\%}$), was 1.21 ± 0.22 for the ins/ins genotype, 1.06 ± 0.27 for the ins/del subgroup and 0.07 ± 0.02 for the del/del genotype. Out of the 19 volunteers, V19 could be considered as a high excretor with mean and median values (3.3 and 3.3) near the WADA threshold of 4 (see Supplementary Table 1). Such case of naturally elevated TG/EG can lead to false estimations and can be attributed either to elevated TG level or to the low excretion of EG [30]. V19 was reported to have naturally elevated TG/EG due to low excretion of EG in urine, according to population reference percentiles, with mean and median concentrations of 13.9 and 13.2 ng/mL (see Supplementary Table 1) [31]. The other volunteers had steroids levels and TG/EG in the general quantile population range, considering their genotype for UGT2B17 [28]. A slight daily variation of the urinary TG/EG was observed (Fig. 3). While the ratio mainly compensate for the daily variation of excretion of TG and EG with low elevation in the morning for the ins/ins and ins/del genotypes, as previously reported [32], a large inter-individual variability of the TG/EG was observed, even among the same genotype subgroup. Finally, the TG/EG ratio was very low for individuals with UGT2B17 del/del genotype due to low urinary excretion of TG (<10 ng/mL) (see Supplementary Table 1). Steroid profile based on population study thus encompassed large inter-individual variations that makes difficult to establish a unique threshold, as already reported by several researches [5,12,15].

3.3. ROC curves analysis

ROC curve analysis was performed to evaluate the overall accuracy of the measured parameters for the discrimination between control and T administered subjects. For that purpose, true positive rate (sensitivity) and false positive rate (1-specificity) were assessed at different thresholds. The accuracy of a decision threshold was evaluated, as each point of the curve corresponded to a sensitivity and specificity couple. For doping control analysis, specificity is particularly important, as less positive cases that

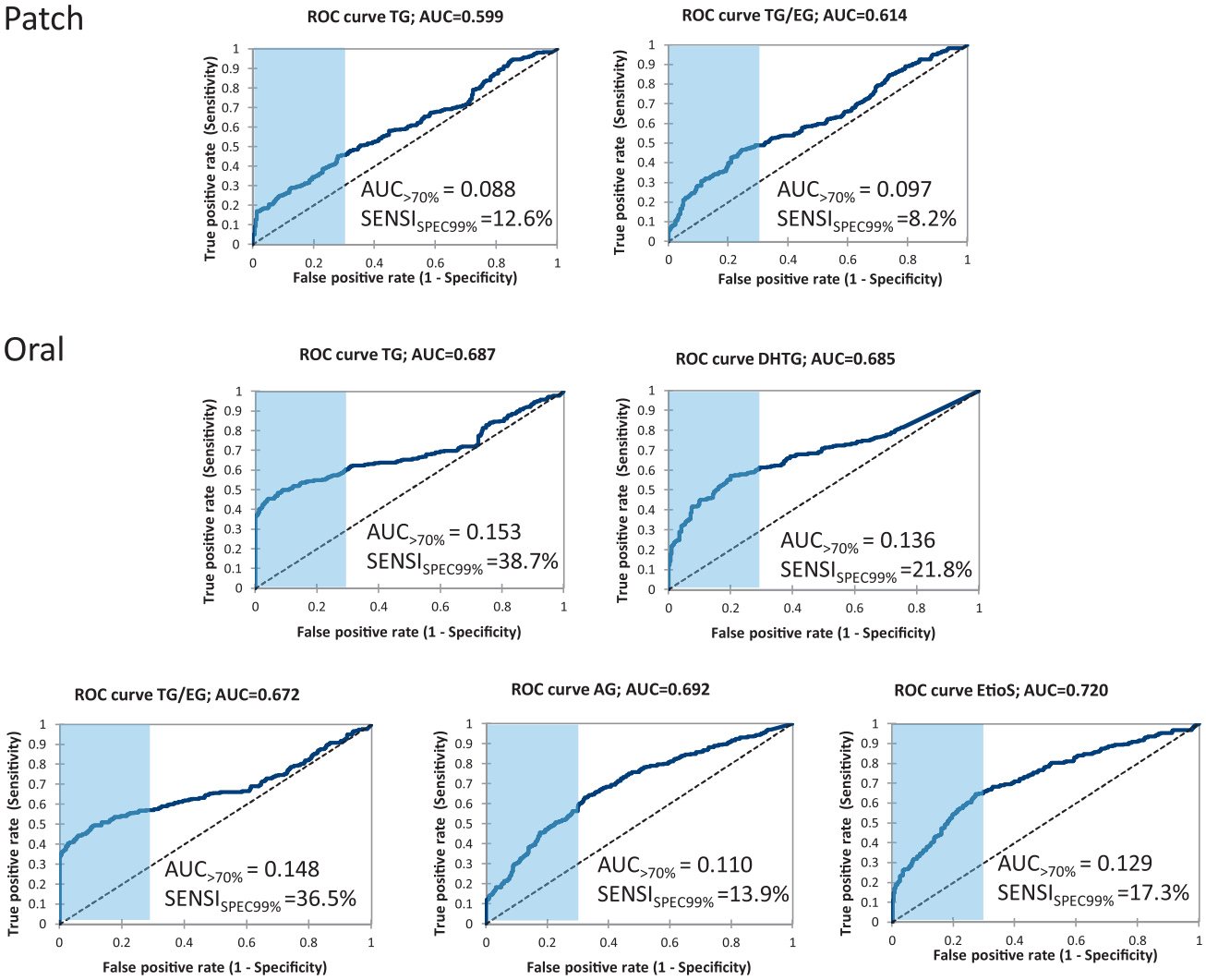

Fig. 4. ROC curves analysis of the most significant steroid profile markers after transdermal T and oral TU administration.

need further confirmation are desired. For screening analysis purpose, the area was computed in the specificity range of 70–100% ($AUC_{>70\%}$) [14,18].

3.3.1. Transdermal testosterone administration

ROC curves were built for each targeted steroid from the control and the patch group. The most relevant markers of the steroid profile were TG and the TG/EG ratio, whose ROC curves are presented in Fig. 4. Regarding topical T application, TG and TG/EG were poorly predictive, with low sensitivity at 99% specificity ($SENSI_{SPEC99\%}$ = 12.6% and 8.2%) and low $AUC_{>70\%}$ = 0.088 and 0.097, respectively. ROC curves of all the other compounds were very close to the diagonal (data not shown), and thus had very low discrimination power. The poor sensitivity could be attributed to the low dose and bioavailability of the topical route, as well as the genotypic UGT2B17 diversity included in the study.

3.3.2. Oral testosterone administration

ROC curves of 5 biomarkers from the steroid profile, including the TG/EG, presented good accuracy indices after TU administration (Fig. 4). As expected, TG and TG/EG provided the best discrimination power ($AUC_{>70\%}$ = 0.153 and 0.148, $SENSI_{SPEC99\%}$ = 38.7% and 36.5%), but parameters such as AG ($AUC_{>70\%}$ = 0.110, $SENSI_{SPEC99\%}$ = 13.9%), as well as DHTG ($AUC_{>70\%}$ = 0.136, $SENSI_{SPEC99\%}$ = 21.8%) showed also satisfactory performance indices.

From the sulfoconjugated steroids, EtioS revealed also appropriate accuracy ($AUC_{>70\%}$ = 0.129, $SENSI_{SPEC99\%}$ = 17.3%). This marker was previously described to be relevant of TU exposure [18], and was expected to be increased especially in del/del genotype for UGT2B17 [33]. To assess the pertinence of each biomarker, individual thresholds were set up, to avoid the influence of ethnicity and genotyping on the reference limit, as proposed for the forthcoming ASP approach.

3.4. Individual threshold reference limits

The usefulness of each marker after transdermal and oral administration of T was evaluated by setting subject-based thresholds. Therefore, the 6 data points collected during the control week (C-t00 to C-t05) were used to define individual thresholds for each volunteer by using the formula [mean ± 3 × SD], as reported in several works [5,11,15]. Detection time was defined as the time after T administration when the measured concentration was higher than the individual threshold, assuming that the administration of T will increase the levels of the metabolites. The detection times for each biomarker and volunteer are presented in Table 3 after the

Table 3
Detection time based on individual threshold [mean control samples ± 3 × SD] for each marker and subject after T transdermal and TU oral administration.

Genotype	Ins/ins							Ins/del							Del/del				
Subject number	3	6	7	11	13	16	18	1	2	4	8	9	10	19	5	12	14	15	17
Patch																			
TG	48; 72	24; 72	8-72	24; 48	8-96	4; 12; 48–60	–	8; 24–72	24–72	24	–	24	72	–	12-96	–	24	–	–
DHEAG	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EG	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AG	–	2; 48–72	–	–	–	–	–	2	48	–	–	–	–	–	72	24–72	–	–	–
EtioG	–	–	–	–	–	–	–	–	48	–	–	–	2–48; 72	–	–	24–72	–	12-24	–
αββ-AdiolG	24–72	–	72	–	–	60–90	2	–	–	–	–	–	–	24	–	–	–	–	–
DHTG	–	–	8	–	–	–	60	–	–	–	–	–	–	–	–	–	2	48–60	–
TS	–	–	–	–	2; 12–24	–	–	–	–	–	–	8	–	–	24; 60–72	12	–	–	–
ES	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DHEAS	–	–	–	–	–	–	–	60	–	–	–	2	–	–	–	60	–	–	60
AS	–	–	2–8; 48	–	–	2	–	–	24–48	–	–	2; 96	–	–	–	60	–	–	–
EtioS	–	2; 8; 12; 60–72	24–60	–	24	–	–	–	–	–	–	8; 60	2; 48	–	–	–	–	–	60
TG/EG	–	4; 24; 60	12; 48; 72	24	4–96	–	8–24; 60–96	8–96	4–12; 60–96	8–24	12–24	72	8; 60–72	12; 72	60	–	24	–	–
TS/ES	–	–	–	–	–	–	–	–	4	–	–	4	4–12	–	–	–	–	4	–
AG/EtioG	–	–	4–12	2; 12; 60	60	–	–	–	4; 12	8	–	–	2–12	–	–	–	12; 48	–	–
AG/EG	48–72	2; 60–96	–	60	48	–	–	2; 48–96	60	60	72	2; 48	24–72	–	–	48–96	48–96	2; 8; 48–96	–
Oral																			
TG	2–12	2–8	2–96	2–12	2–24	2–8; 72	2–8	2–24; 60	2–24; 60	2–4	2–4	2–72	2–8	2–8; 60	4	2	–	2	4
DHEAG	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EG	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AG	–	2–4; 24–72	–	2–8	8	–	–	2	–	–4	2–8	–	2	60	24	2; 24–72	4	2	–
EtioG	24	–	2–4	4; 60	8	–	–	2	–	2–4	2–4	2–4; 24	2; 48; 72	60	–	2–8; 48–72	–	2; 24	4; 72
αββ-AdiolG	2–8; 60–96	2; 24–72	2; 24; 60	2–60	8–96	8; 48–72	24; 72	2; 24; 72	8–60	2	4	2–4	2–8	8; 24; 60–72	–	–	–	2	–
DHTG	2–8	4–8	2–12	2–24	2–12; 48–60	4–8	2–4	2–24; 72	8–12	2–8; 60–72	2–8; 60	2–72	2–8	2–8; 60	–	–	12	48–60	–
TS	–	–	2; 4; 24	–	8–48	2	–	2	–	2	2–8	2–4	–	–	72	2	–	2	4; 8; 60
ES	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DHEAS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AS	–	24–96	48	2	48; 72	2; 48; 72	–	72	24–60	–	4–8	4–72	4; 24–48	60	60	8; 24: 60–72	8; 60; 96	–	4
EtioS	8	2; 8; 24–96	2; 24–60	2–12; 60	8–96	2; 60–72	2	–	60	2–8	2–8	4; 60–96	2–8; 48–72	60	72	2–72	8; 60–72	2–8	2–96
TG/EG	2–12	2–8; 60	2–12	2–8	2–12; 60	2–4	4–24; 72; 96	2–24; 60	4–24; 60	2–4	2–4	2–24; 60	2–8	2–8; 60	–	–	–	–	2
TS/ES	8–12	–	2	8–12	4	–	2–4; 60	–	–	2	12	4	2; 60	–	–	12	–	–	–
AG/EtioG	–	–	–	–	4	–	–	–	–	–	8	–	–	–	60	–	–	–	–
AG/EG	4–12	2–8; 60–96	–	2–8; 60	8	–	–	2	8–12	2–4	2–8	2–12; 60	2–4; 12–24	72	–	2–8; 60	4; 60	2–12	–

administration of 2 consecutive doses of T at 48 h intervals by transdermal and oral route.

3.4.1. Subject-based detectability after transdermal testosterone administration

The transdermal route of administration is particularly interesting for top-level athletes, as peak excretion and T inactivation in the liver can be avoided, while the bioavailability was estimated between 10 and 15% [7]. The pharmacokinetics of T patch system was, to our knowledge, only studied in serum samples from hypogonadal men [34]. In the anti-doping field, alterations of the urinary steroid profile by topical T application were considered after T gel administration but the interpretation was only based on GC–MS and GC–IRMS measurements [8,9,35]. Slight increases of urinary TG and TG/EG were observed after single T gel application, whereas the increase of 5α-metabolites levels, including AG and 5ααβ-AdiolG, due to high 5α-reductase activity in the skin was only observed after seven 100 mg T gel applications per week [8].

During the second week of the clinical trial, the volunteers received 2 patches of 2.4 mg/24 h twice a week alternatively on the left and right shoulder, on Monday and Wednesday and the transdermal system was kept for 48 h. Blood and urine samples were collected at 0 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 60 h, 72 h and 96 h (P-t00–09), while the patches were administered at 0 h and 48 h (P-t00 and P-t06). As the WADA-based population threshold of 4 was never reached for the TG/EG (see Supplementary Table 1), this marker could not be used to highlight T patch doping. Individual thresholds are expected to be most powerful strategy to detect low T transdermal dose administration. As reported in Table 3, only a few markers revealed detection ability based on individual threshold. While the concentration of EG, DHEAG and ES never rose beyond the individual threshold, the best markers were TG, the TG/EG ratio and the AG/EG ratio. Individual thresholds were reached for 13 volunteers out of the 19, and the effect was less pronounced for the del/del genotype, as expected. The elevation of those markers after T patch administration followed the slow-kinetic release of the topical route with detection time mostly between 8 h and 24 h after T patch administration.

The other targeted steroids quantified in the urinary samples were not notably modified after T patch administration. This can be attributed to the low dose and bioavailability of 2.4 mg/24 h in combination with the slow-releasing kinetics of topical route [9]. 5α-metabolites, such as AG and 5α-AdiolG were reported to be increased after T transdermal application due to high 5α-reductase activity in the skin [36]. It is worth mentioning that traditional 5ααβ-AdiolG and 5βαβ-AdiolG metabolites of T were not followed in the present study, as the corresponding standards were not at disposal during the validation process. Thanks to the generic UHPLC–QTOF-MS^E platform, the chromatographic peaks related to the metabolites could be extracted and compared with reference standards afterwards. By these means, three isomers were identified at m/z 467.2645 ± 0.02 at retention time (RT) 15.83, 16.43 and 17.38 min, corresponding to 5βαβ-Adiol-3-O-G, 5ααβ-AdiolG and 5βαβ-Adiol-17-O-G, respectively. By plotting the area of those metabolites to the DHTG-d_3 IS, empirical individual threshold were set for those metabolites and ratio between them (see Supplementary Table 2). The detection time for those parameters were evaluated and found to be higher than the individual threshold only by few volunteers out of the 19, with the most sensitive markers being 5ααβ-AdiolG and the ratio 5ααβ-AdiolG to 5βαβ-Adiol-17-O-G. These results thus corroborated the elevation of 5α-metabolites after transdermal administration of T due to higher 5α-reductase activity in the skin, but only for some subjects, independently of the UGT2B17 genotype.

As an example, the detection times based on individual threshold levels are presented in Fig. 5 for 3 volunteers and 3 significant biomarkers after T administration. After T patch administration the TG/EG ratio rose above the individual threshold for subject 01 and 13, even if the WADA threshold of 4 was never reached. However, for the subject 15 bearing a deletion enzyme polymorphism, the TG/EG ratio remains far below the individual threshold. Among the 3 selected subjects, the profiles were different for the biomarker DHTG. Indeed, while the concentration of DHTG never reached the individual threshold for both homozygotous and heterozygotous inserted genotypes, it was reached for the deleted genotype at time 60 h and 72 h. However, none of the other individuals bearing the deletion genotype presented significant elevation of DHTG after T transdermal administration, thus revealing the important heterogeneity in metabolism even among a same genotype subgroup. The analyte EtioS was also presented in Fig. 5, as this compound might be of complementary information especially for deleted genotype [33]. Following T patch administration, this biomarker only rose slightly beyond the individual threshold for subject 13, 24 h after the administration. EtioS was an interesting marker only for few volunteers and especially subject 06 (Table 3), for whom the individual threshold was reached at several times.

While the classic biomarkers used to screen for T misuse were not able to detect low T transdermal dose administration, the longitudinal follow-up of these markers based on subject-based thresholds presented higher detection ability predominantly at time 24 h after patch application. However, most of the parameters remain below the individual threshold for the individuals bearing the del/del genotype.

3.4.2. Subject-based detectability after TU pill administration

The metabolism of endogenous steroids was evaluated in this research after 80 mg oral TU pill intake for the 19 volunteers. Excretion kinetics of glucuroconjugated metabolites after oral TU intake was largely studied in young healthy male after single dose and long-term administration [36]. Rapid and elevated increase of TG/EG and TG are well known indicators of TU misuse in sport and were also observed in this study. As expected, the TG/EG WADA threshold of 4 was rapidly reached 2 h post oral intake by ins/ins (mean 13.73 ± 7.84) and ins/del (mean 31.70 ± 31.73) genotypes, while the ratio was not affected by del/del genotypes. Considering subject-based strategy, the most relevant biomarkers, according to their rise above the individual threshold were glucuroconjugated analytes including TG, AG, EtioG, 5αββ-AdiolG, DHTG, as well as the ratios TG/EG and AG/EG. However, TG, 5αββ-AdiolG, DHTG, TG/EG and AG/EtioG ratios could not detect exogenous intake of TU by individuals bearing the del/del genotype (Table 3). Indeed, while 5αββ-AdiolG and DHTG were sensitive markers, their concentrations were under the limit of detection (LOD) for subjects bearing the enzyme deletion (see Supplementary Table 1). For all of the elevated markers and in accordance with several studies, the detection window ranged from 2 h to 12 h, with an excretion peak between 4 h and 12 h and returned to the baseline level 24 h after pill intake [3,5,17,28]. Fig. 5 presented the individual monitoring of 3 relevant biomarkers for 3 different genotype subjects. After oral TU administration, the profile differed between individuals for each biomarker. Indeed, the TG/EG ratio presented a rapid and acute raise lasting from 2 to 12 h after the first oral dose, and was also increased after the second dose by the inserted genotypes, while it was not affected for subject 15, bearing the UGT2B17 del/del genotype. A similar profile was observed for DHTG, but this biomarker was also modified for the individual devoid of UGT2B17 enzyme, meaning that the glucuroconjugation also occurred via another UGT enzyme isoform, as reported elsewhere [37].

The evaluation of other biomarkers would be useful to enhance the detection window. Several researches studied the sulfate metabolism after TU ingestion, with the hypothesis that sulfoconjugated metabolites shall be relevant for complementary information

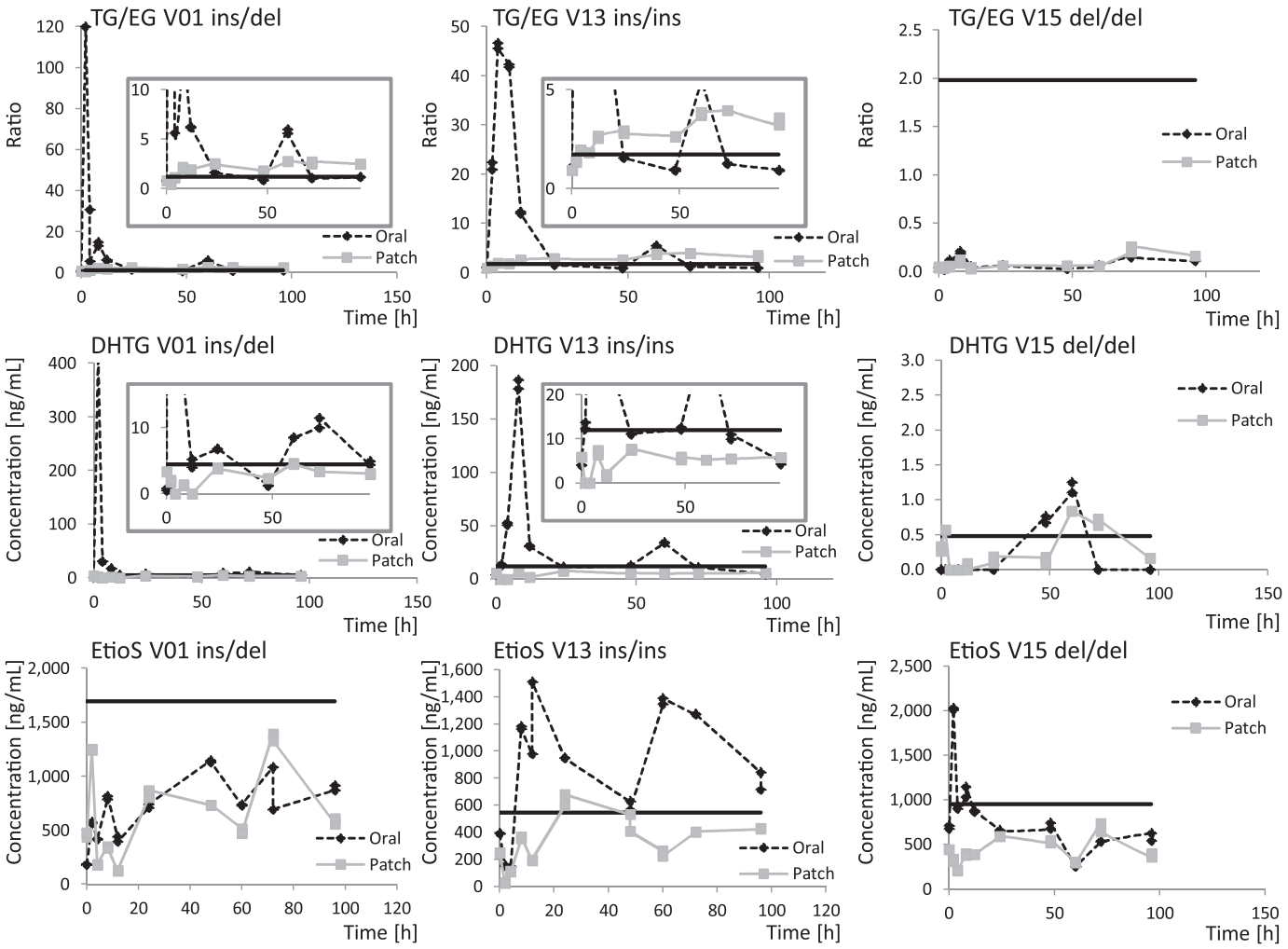

Fig. 5. TG/EG, DHTG and EtioS excretion profiles. Subjects 01, 13 and 15 are depicted with the individual threshold (bold line) after transdermal T and TU oral administration.

especially for individuals devoid of the UGT2B17 enzyme [33] or for subjects with chronic elevated TG/EG [38–40]. AS and EtioS were markers of interest of TU intake, while TS and the ratio TS/ES did not present interesting detection ability (Table 3). AS and EtioS were particularly interesting as at least one detection time could be observed for each of the del/del genotypes. This is in accordance with previous results revealing the potential use of EtioS as a complementary marker after oral TU administration [18]. This marker was increased by most of the volunteers at time 60 h and 72 h, meaning 12–24 h after the second oral dose administration. As presented in Fig. 5, EtioS profile was very heterogenous for the 3 subjects. However, according to subject V13 profile, a longer detection window than based on the TG/EG ratio could then be expected by following this marker, as also shown in Table 3. Eventually, the concentrations of EG, ES, DHEAG and DHEAS were not affected following oral TU administration with respect to the study cohort. Consequently, AS and EtioS could be used as complementary biomarker to screen for exogenous administration of TU. Sulfoconjugated steroids might be integrated in the steroid profile for longitudinal ASP follow-up methods, especially for their relevance regarding UGT2B17 del/del individuals.

The effect of TU intake was also assessed for the 5α/β-AdiolG isomers, which are common markers as part of the steroid profile to assess for exogenous steroid ingestion [1,41]. It is noteworthy that the individual monitoring of these compounds was based on peak area ratio to the DHTG-d_3 IS, as the standards were not at our disposal when the analyses were performed. 5βαβ-Adiol-3-O-G was not affected by the oral ingestion of TU, while interestingly the concentrations of 5βαβ-Adiol-17-O-G and 5ααβ-AdiolG isomers were significantly affected. Finally, ratios between AdiolG isomers were evaluated and the ratio 5βαβAdiol-3-O-G/5ααβAdiolG was relevant also to detect exogenous oral intake of TU by the del/del genotype, while the ratio 5βαβAdiol-3-O-G/5βαβAdiol-17-O-G revealed detection ability for both homozygotous and heterozygotous inserted genotype.

Based on individual threshold strategy, the integration of multiple parameters in a longitudinal follow-up could enhance the detection ability and discrimination power between confounding factors, individual biological variability and oral TU intake doping.

3.5. Other relevant biomarkers

A prior study reported the application of the method developed on the previous generation QTOF to urine samples from a clinical study including 17 healthy male volunteers and the administration of placebo or TU pills. A steroidomic approach applied to the samples of this previous clinical trial revealed 3 metabolites as potential markers of TU intake, including DHTG, an isomer of either AS, DHTS or EtioS and a hydroxylated form of AG, DHTG or EtioG [18]. The identification of DHTG, thanks to comparison with a reference standard, highlighted the relevance of the untargeted steroidomic approach since DHTG is a well-known and sensitive

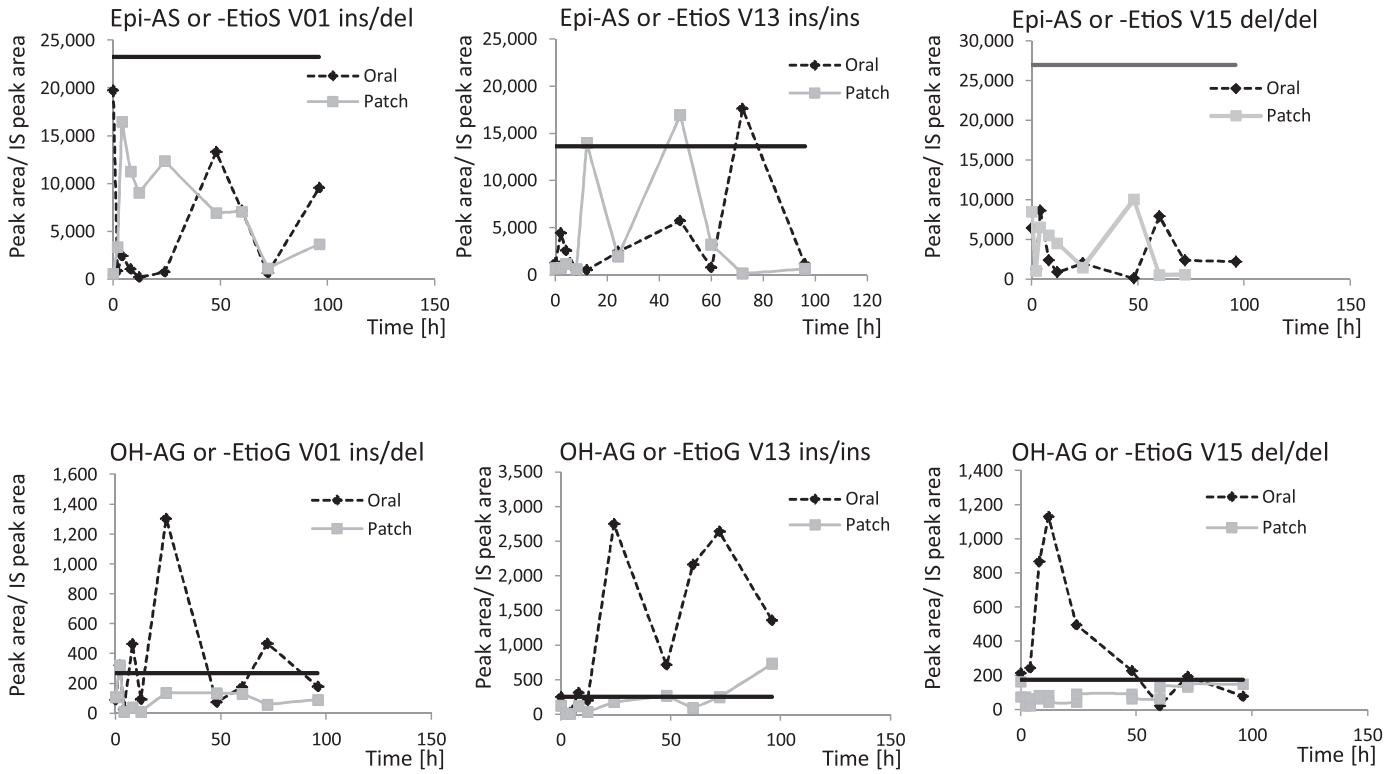

Fig. 6. Excretion profiles for a sulfo-conjugated isomer of AS or EtioS and a hydroxy metabolite of AG or EtioG. Subjects 01, 13 and 15 are presented with the individual threshold (bold line) after transdermal T and oral TU administration.

biomarkers, which is included in the routine steroid profile [42,43]. As already mentioned, DHTG was thus included in the quantitative method with its isotopically labeled IS DHTG-d_3. As no reference standard was available for the two other biomarkers, their chromatographic peaks corresponding to the given *m/z* and RT couple were extracted from the first MS trace and integrated. To overcome the analytical variability, a correction was made with a selected deuterated IS having the narrowest *m/z* and RT (AG-d_4 for the hydroxylated form of AG, EtioG or DHTG; and AS-d_4 for the epimer of AS, EtioS or DHTS). Fig. 6 presented the excretion profile of the sulfo-conjugated epimer and the hydroxy-metabolites with the individual threshold from 3 volunteers out of the 19, after transdermal and oral T administration. The sulfoconjugate epimer of AS or EtioS, which is probably epiandrosterone sulfate, according to the literature, did not reveal high detection ability, and only rose beyond the individual threshold for few volunteers. Indeed, as presented in Fig. 6, the concentration of this metabolite was elevated only by subject V13 out of the 3 subjects shown. This metabolite was increased in urine after the administration of 5αββAdiol, a prohormone of DHT [44] and was also reported to be a relevant marker after DHEA administration [38]. Nevertheless, this metabolite seemed not to be the preferentially way of elimination after T administration. Hydroxy isomer of AG, EtioG or DHTG presented higher detection ability among the study cohort. This isomer could be potentially 6-β or 16-α hydroxy AG or EtioG, with respect to literature [28]. As presented in Fig. 6, while the level of this metabolite was not significantly modified after T transdermal administration, the individual threshold was reached for all of the volunteers after 80 mg oral TU ingestion. A raise in the excretion of this biomarker was reported from 12 to 24 h after intake for all UGT2B17 genotypes, and the increase was even more marked 12 h after the second oral dose (60 h). This metabolite thus seemed to be a promising marker for long-term TU detection and particularly after multiple doses administration. This corroborated previous study revealing an extended detection window for hydroxy metabolites of T when compared to TG/EG [5]. Since levels rose above the individual threshold also for the individual bearing the del/del genotype, this suggested that this metabolite was not predominantly glucuroconjugated by UGT2B17. Hydroxy-metabolites constitute therefore promising biomarkers to screen for the long-term detection of TU intake, even for individuals having a del/del UGT2B17 genotype. However, as the results were based on peak area ratio to a deuterated IS, without using calibration curves and proper validation, the quantitative estimation may be less accurate. Stereoselective synthesis of this product, which might be available in the near future, will be very relevant for its precise structural and proper quantitative evaluation and integration in the ASP.

4. Conclusion

Thanks to the new generation UHPLC–QTOF-MS^E platform used in this research, excellent qualitative and quantitative results were obtained with high mass accuracy and sensitivity. The method allowed the quantification of 12 targeted glucuro- and sulfo-conjugated steroids in more than 500 urine samples. Phase II metabolism information was studied on samples arising from a clinical trial involving 19 healthy male volunteers after the administration of transdermal and oral T. Nonetheless, urine analysis of steroids suffers from large inter-individual variability including genetic polymorphism, diuresis and circadian rhythm. While UGT2B17 polymorphism was highly linked to TG excretion in the presented results, the UGT2B15 polymorphism could not be highlighted to influence specific metabolites excretion, as it does not lead to the absence of enzymatic activity. Therefore, due to the complexity to integrate those data, longitudinal follow-up of individuals was the strategy of choice. This is particularly important, because classical criteria based on the urinary steroid profile are not adequate to reveal T misuse via topical route, and the detection

of administration of high oral dose is limited to short time post dose.

Based on this approach, transdermal T application consisting in low dose and slow-releasing kinetic could be detected at several time points by following TG or the ratios TG/EG and AG/EG. After 80 mg oral TU ingestion, the sulfoconjugated analyte EtioS, which was not screened by GC–MS approaches, was revealed as a promising biomarker, especially for individuals having a deleted UGT2B17 genotype. Additionally, hydroxy metabolites were highlighted as potential long-term detection markers of TU. The results presented in this research, confirmed this hypothesis, with an increase of this marker after TU intake even for individuals bearing the del/del genotype. Subject-based references as found in the ASP, implemented with novel biomarkers (hydroxy, sulfoconjugated) would thus be the most adequate tool to screen for T misuse.

Eventually, the blood matrix collected during this clinical trial will constitute invaluable complementary information to reveal T doping as future perspective. In addition, as an untargeted acquisition over the entire selected mass range was possible with the QTOF mass spectrometer, unknown markers could be extracted from the first MS function, based on their RT and *m/z*. In the future, the predictive ability of these markers will be investigated with respect to T transdermal and oral administration, without necessity to reanalyze the samples. The selection of untargeted metabolites is promising for a broader steroid profiling on fresh and well-structured clinical study samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2013.05.018.

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