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PAPER IN FOREFRONT

Longitudinal monitoring of endogenous steroids in human serum by UHPLC-MS/MS as a tool to detect testosterone abuse in sports

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Abstract The detection of testosterone abuse in sports is routinely achieved through the 'steroidal module' of the Athlete Biological Passport by GC-MS(/MS) quantification of selected endogenous anabolic androgenic steroids (EAAS) from athletes' urines. To overcome some limitations of the "urinary steroid profile" such as the presence of confounding factors (ethnicity, enzyme polymorphism, bacterial contamination, and ethanol), ultrahigh performance liquid chromatography (UHPLC) measurements of blood concentrations of testosterone, its major metabolites, and precursors could represent an interesting and complementary strategy. In this work, two UHPLC-MS/MS methods were developed for the quantification of testosterone and related compounds in human serum, including major progestogens, corticoids, and estrogens. The validated methods were then used for the analyses of serum samples collected from 19 healthy male volunteers after oral and transdermal testosterone administration. Results from unsupervised multiway analysis allowed variations of target analytes to be assessed simultaneously over a 96-h time period. Except for alteration of concentration values due to the circadian rhythm, which concerns mainly corticosteroids, DHEA, and progesterone, significant variations linked to the oral and transdermal testosterone administration were observed for testosterone, DHT, and androstenedione. As a second step of analysis, the longitudinal monitoring of these biomarkers using intra-individual thresholds showed, in comparison to urine, significant improvements in the detection of testosterone administration, especially for volunteers with del/del genotype for phase II UGT2B17 enzyme, not sensitive to the main urinary marker, T/E ratio. A substantial extension of the detection window after transdermal testosterone administration was also observed in serum matrix. The longitudinal follow-up proposed in this study represents a first example of 'blood steroid profile' in doping control analysis, which can be proposed in the future as a complement to the 'urinary module' for improving steroid abuse detection capabilities.

Keywords Testosterone doping · Serum · Steroid profile · UHPLC-MS/MS · Multiway data analysis

Introduction

Testosterone (T) is a hormone secreted by endocrine glands and constitutes the key male endogenous anabolic androgenic steroid (EAAS). It promotes growth of skeletal muscle, stimulates erythropoiesis, and mediates secondary sexual characteristics, while influencing behavior and cognition [1, 2].

For doping practices, the use of T at high doses is mainly interesting for its anabolic action in strength sports, whereas in endurance sports, low but frequent doses are preferred to improve recovery, limiting the myotrophy effect [3]. T misuse is currently evaluated by anti-doping laboratories by measuring selected endogenous steroid hormones in urine by GC-MS

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and/or GC-MS/MS. This strategy includes quantification of T, its precursor dehydroepiandrosterone (DHEA), its epimer epitestosterone (E), and its most abundant metabolites, such as dihydrotestosterone (DHT), androsterone (A), etiocholanolone (Etio), 5α-androstanediol (5α-Adiol), and 5β-androstanediol (5β-Adiol). These markers are extensively metabolized and excreted as glucuroconjugated compounds in urine. To date, the most sensitive and specific criterion applied in routine control is a proportional relationship between two of these parameters (i.e., the T/E ratio) [4]. Based on population reference intervals, a urinary T/E>4 threshold was employed until a couple of years ago to declare a test suspicious, followed by confirmation analysis by GC-C-IRMS [5]. However, T/E biomarker was found to be subjected to large inter-individual variability, influenced by gender, route of administration, diet, physical exercise, and particularly by genetic polymorphism [6]. In this context, enzyme polymorphism of UGT2B17, the major UGT isoform responsible for T glucuronidation, largely contributes to the explanation of the T/E variability [7], as individuals with a homozygous deletion of UGT2B17 (del/del) present very low levels of T glucuronide in urine. Moreover, it was also reported that 40 % of the individuals devoid of this enzyme did not show noticeable elevation of T/E after exogenous T administration, making detection of T abuse very difficult for this genotype [8–10].

To improve capabilities of T misuse detection by limiting inter-individual variability of biomarkers, a "urinary steroidal module" has been implemented in the Athlete Biological Passport, starting from January 1, 2014 [4]. Using this approach, T/E as well as other three metabolites ratios (e.g., A/T, A/Etio, 5α-Adiol/5β-Adiol) are monitored over time for each athlete using "intra-individual reference values" generated by a Bayesian Adaptive Model [11]. With this individual monitoring, it was recently observed that the detection window of oral T administration in urine was mainly included between 2 and 12 h; moreover, for transdermal T administration, which could never be revealed by T/E>4, detection windows mostly between 8 and 24 h were reported, in agreement with the slow-kinetic release of the topical application. Nevertheless, for both administration routes, volunteers devoid of UGT2B17 enzyme almost never exceeded individual thresholds, with sporadic exceptions at 2 to 4 h for oral and 24 h for transdermal administrations, respectively [12].

Nowadays, the urinary longitudinal monitoring has improved the capability of detecting steroids misuse [13] but this methodology still suffers from the presence of confounding factors in urine matrix both endogenous (e.g., enzyme induction and inhibition) and exogenous (medications, bacterial contamination, ethanol, etc.) [14]; hence additional approaches and markers of exogenous T administration are still required. In this context, steroids analysis in blood could represent an interesting strategy complementary to the urinary 'steroid profile.' Indeed, this matrix is less subjected to risk of manipulation and/or contamination and it can be assumed that for compounds circulating in blood, the dose to concentration profiles can be accurately estimated for pharmacokinetics interpretation. Furthermore, the investigation of steroid metabolism and bioavailability of pharmaceutical preparations in blood is also particularly relevant. In this context, several studies for doping control have already reported biomarkers of interest in blood after oral administration of esterified T by means of immunoassay, radioimmunoassay, or LC-MS/MS [15–17]. The latter technique is preferred today as it guarantees suitable sensitivity and optimal quantitative performances; moreover it requires an easier and faster sample preparation compared with GC analysis as no derivatization is needed [18].

In this research, UHPLC-MS/MS analyses were performed for the quantification of 14 steroid hormones in serum, including major circulating progestogens, androgens, corticoids, and estrogens. Then, 532 serum samples collected during a clinical trial involving 19 healthy male volunteers who were administered with transdermal T and oral T undecanoate (TU) were analyzed. To better understand the modifications of blood steroids levels induced by an exogenous T administration, acquired data were investigated through unsupervised multiway analysis with the aim of highlighting promising biomarkers for a tentative longitudinal follow-up in serum.

Materials and methods

Clinical study and preliminary analyses

All samples came from a clinical trial involving 19 healthy male volunteers, aged 19 to 28 y (mean 24.3±2.7 y) with a body mass index (BMI) between 18.3 and 27.2 (mean 23.1± 2.4 kg/m^2), as fully described in [12]. 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 no. 155/11). The clinical trial involved four major phases: for each volunteer, the first wk was dedicated to the collection of control samples (no T administration) at different time points, and the second wk coincided with the administration of two T transdermal patches (Testopatch 2.4 mg/24 h; Pierre Fabre Pharma GMBH, Freiburg, Germany) twice, involving 4.8 mg/24 h administration, with 48 h intervals; the third and fourth wk corresponded to a wash-out period. Finally, two oral pills of 40 mg TU each (Andriol Testocaps; Essex Chemie AG, Luzern, Switzerland) were given twice during the last wk of the trial with a 48-h interval. A total number of 532 whole blood, serum, and urine samples were collected corresponding to defined collection time points over a period of 24 h (C0 to C5) for the control group and over a 96-h follow-up period for

the transdermal (P0 to P09) and oral administration of T (O0 to O9).

Serum samples were centrifuged at 1500×*g* for 15 min after 15 min stabilization at room temperature, and aliquots of approximately 500 μL were prepared and then stored at –20 °C until analyses.

Luteinizing hormone (LH) and sex hormone-binding globulin (SHBG) concentrations were determined with an Immulite 2000 (Siemens, Munich, Germany). LH and SHBG quality controls were obtained from BioRad Clinical Diagnostics (Hercules, CA, USA). Finally, the genotype for the genes coding for the enzyme UGT2B17 was established on the whole blood samples from all the participants of the clinical study as described elsewhere [12]. Analysis resulted in 7 ins/ins (V3, V6, V7, V11, V13, V16, V18), 7 ins/del (V1, V2, V4, V8, V9, V10, V19) and 5 del/del (V5, V12, V14, V15, V17) volunteers.

UHPLC-MS/MS analyses

Chemicals and reagents

T certified solution (5 mg/mL) was purchased from Lipomed (Arlesheim, Switzerland), E certified solution (5 mg/mL) from Cerilliant (Round Rock, TX, USA), androstenedione, DHEA, estrone, estradiol, and estriol from Steraloids (Newport, RI, USA), DHT from Chemie Brunschwig (Basel, Switzerland), progesterone from Laboratoire Golaz (Lausanne, Switzerland), 17α-hydroxyprogesterone, corticosterone, cortisol, and estradiol-$^{13}C_3$ as well as deoxycorticosterone and 11-deoxycortisol certified solutions from Sigma Aldrich (Buchs, Switzerland). All other labeled internal standards (IS) were provided by NARL (Sydney, Australia). Methanol (MeOH) was purchased from Macron Fine Chemicals (Deventer, The Netherlands), dichloromethane (DCM) from VWR International (Fontanay-sous-Bois, France), acetonitrile (ACN) ULC/MS (>99 %), and formic acid (FA) ULC/MS (99 %) were supplied by Biosolve BV (Valkenswaard, The Netherlands), while ammonium hydroxide solution 30 % from Sigma Aldrich (Buchs, Switzerland). Charcoal Dextran Stripped Human Serum was obtained from Dunn Labortechnik GmbH (Asbach, Germany). Deionized water was obtained by a Milli-Q-grade system (Millipore, Billerica, MA, USA) and was used for the preparation of all LC mobile phases.

Stock solutions of each analyte and IS were prepared at a concentration of 1 mg/mL in MeOH; intermediate solutions at appropriate concentrations were prepared through consecutive dilution of the stock solutions in MeOH. Analyte mixture solutions (working solutions) were then prepared in MeOH and used for preparation of calibration and validation samples in depleted serum. A mixture of IS (IS-mix) was prepared by spiking different volumes of each IS intermediate solution at appropriate concentrations for each IS in MeOH (see Electronic Supplementary Material (ESM) Table S1 for concentration details). All stock, intermediate, and working solutions were maintained at –20 °C in 10 mL glass tubes.

Sample preparation

Supported liquid extraction (SLE) on ISOLUTE SLE+ (Biotage, Uppsala, Sweden) 400 μL 96-well plates was used to extract steroid hormones from serum samples. For each sample, 200 μL of serum were spiked with 20 μL of the IS-mix, diluted with 200 μL of water, and then agitated for 15 min at 250 rpm. Each well was then loaded with 400 μL of each serum sample and positive pressure of 3 psi was applied for 30 s to facilitate sample loading and adsorption; the elution was carried out, after a 5 min waiting period, by adding 700 μL of DCM to each well and applying a pressure of 3 psi for 1 min. Extracts were collected in collection plates equipped with 1.5 mL glass inserts, evaporated for approximately 15 min at 40 °C under a stream of nitrogen, and finally reconstituted with 100 μL of a MeOH-H_2O 50:50 (v/v) solution (reconstitution solvent). After 15 min of gentle shaking (250 rpm), 10 μL of each extract were injected into UHPLC-MS/MS for analyses.

Androgens, progestogens, and corticoids analysis

Analyses were performed using a UPLC coupled to a Xevo-TQ S triple quadrupole MS/MS system from Waters (Milford, MA, USA). Data were processed using MassLynx PC software (version 4.1) and TargetLynx application manager. Liquid chromatography was performed using an Ethylene Bridged Hybrid (BEH) C_{18} column (100×2.1 mm, 1.7 μm; Waters) set at 30 °C. The mobile phase A was a solution of 0.1 % FA in H_2O and the mobile phase B was 0.1 % FA in ACN. The gradient started linearly from 2 % to 25 % B over 0.5 min, followed by an increase to 58 % B over 5.5 min, and by a further increase to 98 % B over 2 min; the column was then re-equilibrated for 3 min at initial conditions. The injected volume was 10 μL and the flow rate was set at 400 μL/min.

ESI-MS/MS analysis was performed in positive mode. Capillary voltage was set at 3.0 kV and source temperature was maintained at 150 °C. Desolvation gas temperature and flow rate were set at 550 °C and 1200 L/h, respectively. Cone gas flow rate was set at 150 L/h and nebulizer gas flow at 5 bar. Collision gas flow was maintained at 0.15 mL/min of argon. Cone voltage, collision energy, and MS/MS transitions were optimized by infusing standard solutions of each compound at 100 ng/mL in reconstitution solvent (see Table 1).

Estrogens analysis

The same instrument and analytical conditions were used for the analysis of estradiol, estrone, and estriol, except for the

Table 1 MS/MS transitions and parameters

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Testosterone	289.2	97.1 ; 109.1[a]	28	18 ; 22
Testosterone-d3	292.3	97.1	20	20
Epitestosterone	289.2	109.3[a] ; 97.1	34	22 ; 18
Epitestosterone-d3	292.3	97.1	42	20
Androstenedione	287.4	97.1 ; 109.1[a]	44	26
Androstenedione-d7	294.2	113.1	42	26
Progesterone	315.3	97.1[a] ; 109.1	28	22 ; 28
Progesterone-d9	324.4	100.1	16	18
17αOH-progesterone	331.2	109.1[a] ; 97.1	22	22 ; 28
17αOH-progesterone-d8	339.3	100.1	26	24
DHEA	271.3[c] ; 289.3	253.2	32	12
DHEA-d5	276.2[c]	258.2	8	14
DHT	291.4	159.2 ; 255.2[a]	42	20 ; 14
DHT-d3	294.3	258.3	36	14
Corticosterone	347.4	91.1 ; 121.1[a]	40	44 ; 24
Corticosterone-d8	355.3	125.1	12	22
Cortisol	363.4	91.1[a] ; 121.1	38	25 ; 26
Cortisol-d4	367.0	121.0	38	25
Deoxyorticosterone	331.3	97.1[a] ; 109.1	48	22 ; 24
Deoxyorticosterone-d8	339.3	113.1	48	22
11-Deoxycortisol	347.3	97.1[a] ; 109.1	26	22 ; 24
11-Deoxycortisol-d2	349.0	97.0	20	38
Estrone	269.0	144.9[a] ; 182.9	32	38 ; 36
Estriol	287.0	144.9 ; 170.9 [a]	54	42 ; 34
Estradiol	271.0	145.0 [a] ; 182.9	24	36 ; 34
Estradiol-13C_3 [b]	274.0	148.0	20	38

[a] Transition used for quantification.

[b] Used as IS for all estrogens.

[c] Precursor ions with loss of H_2O.

mobile phases and the ESI ionization mode. Mobile phase A was H_2O and mobile phase B was pure ACN. Post-column infusion with 0.5 % NH_4OH in H_2O at 5 μL/min was performed and MS/MS analysis was carried out in ESI negative mode using the same source parameters described above, except for the capillary voltage, set at –1.5 kV. Estrogens and IS were monitored using the MS/MS conditions described in Table 1.

Methods validation

Methods described above have been validated in compliance with International Standards for Laboratories of the World Anti-Doping Code [19] and ISO 17025. Different parameters such as selectivity and quantitative performances (trueness, repeatability, intermediate precision, linearity range, limit of quantification) were evaluated. Selectivity was assessed by extracting and analyzing six depleted serum samples (three different aliquots of two different pools of human charcoal stripped serum, negative control) and six serum samples spiked with a low concentrated analyte mix solution. Quantitative validation was performed on three different analytical series using spiked depleted serum samples. For each series, eight calibration and eight validation samples were prepared in duplicate and quadruplicate, respectively, in depleted serum at appropriate concentrations for each compound (concentration details in ESM Table S2). Trueness, repeatability, intermediate precision, linearity range, and limit of quantification (LOQ) were determined for each analyte at each concentration level of the validation standards.

Clinical study samples data treatment

With the aim of highlighting promising blood biomarkers of T administration, parallel factor analysis (PARAFAC) multiway data treatment was performed on the dataset obtained from the

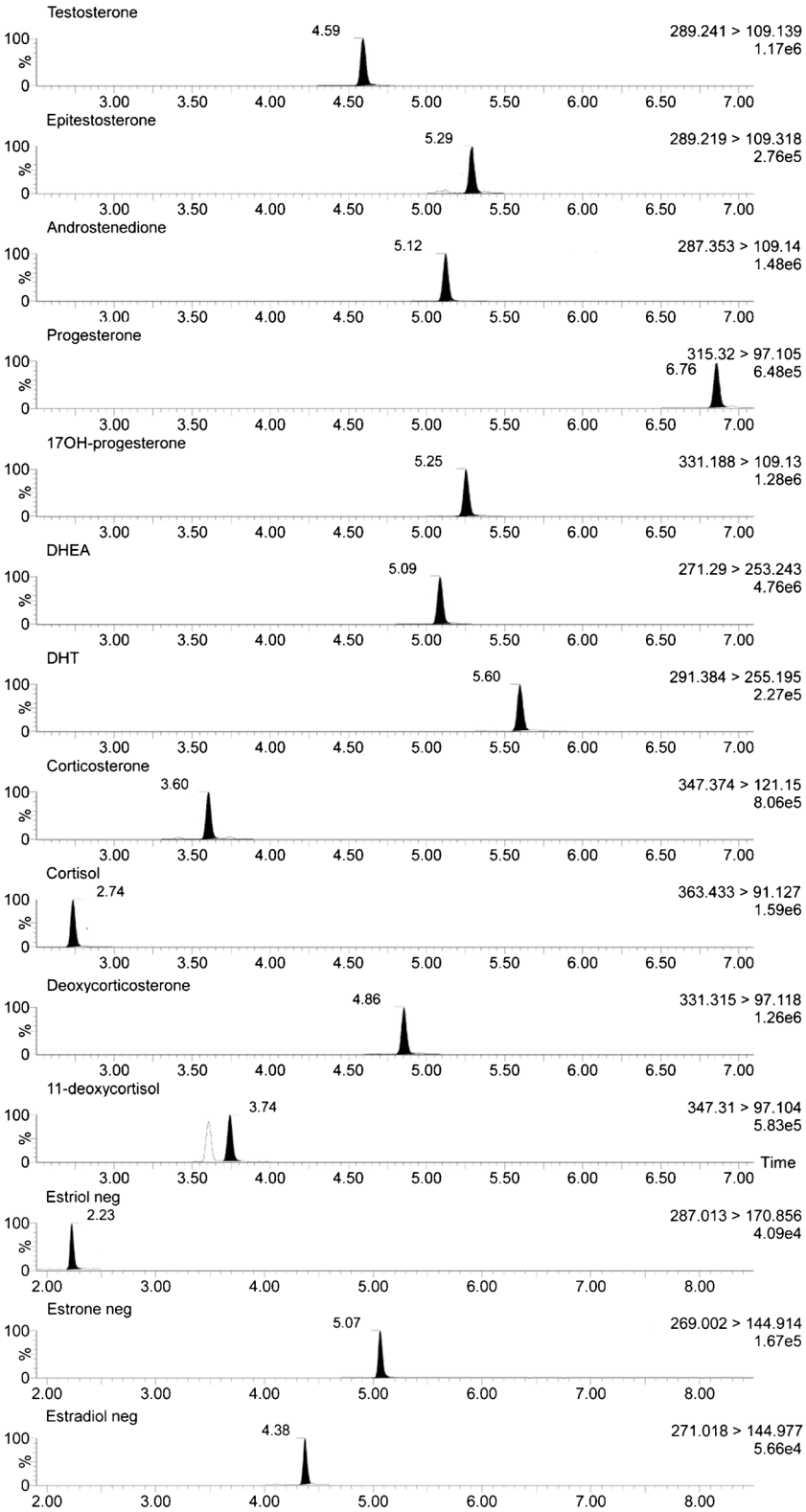

Fig. 1 Chromatograms of an extracted calibration sample

analyses of clinical study samples. PARAFAC models were computed under the MATLAB 8 environment (The MathWorks, Natick, MA, USA) with routines implemented in the N-Way Toolbox (version 3.1) [20]. The CORCONDIA criterion was used to assess model size [21].

Results and discussion

Methods development

The setup of a single method for the simultaneous analysis of all target androgens, progestogens, corticoids, and estrogens represented the first challenge faced during method development. Primarily, a single LC-MS method using ESI polarity switching mode (negative ionization needed for estrogens and positive ionization for all other analytes) was tested, but performances were judged unsatisfactory, especially for estrogens quantification in low pg/mL range. Thus, the development of two separated methods requiring distinct positive and negative ionization, respectively, was preferred. In ESI negative mode, the optimization of LC mobile phases and the addition of a post-column infusion of 0.5 % NH_4OH in water both contributed to a significant improvement of quantitative performances for estrogens. In ESI positive mode, the main issue in method development was attributable to the necessity of monitoring a wide range of concentrations, from few pg/mL (e.g., E and progesterone) to several hundreds of ng/mL (e.g., cortisol). Additionally, for some hormones such as T, it was also necessary to consider large differences of reference concentrations in serum between male and female. An acceptable balance was found thanks to the high sensitivity and large dynamic range, corresponding to approximately three orders of magnitude, of triple quadrupole used in this study.

An example of chromatograms obtained for an extracted calibration sample is shown in Fig. 1. A satisfactory chromatographic separation between all target steroid hormones was obtained. This was important especially in the case of 11-deoxycortisol and corticosterone, which had the same precursor ion mass, similar fragmentation patterns, and close retention times.

In addition to LC-MS, a SLE procedure was set up for the extraction of steroid hormones from serum, allowing high extraction recoveries and clean extracts. In the SLE, compounds are separated through their different affinities for a diatomaceous earth based stationary phase through which the sample is percolated. The process is analogous to traditional liquid–liquid extraction (LLE) and includes, after analytes adsorption, the use of the same organic solvents for analyte elution. Furthermore, this extraction procedure, contrary to LLE, has also the advantage of eliminating problems such as emulsion formation, increasing analyte recoveries and being easily automated in 96-well plates configuration for high-throughput (2 to 3 h for approximately 80 samples). DCM was chosen as elution solvent because of its low polarity, which is well adapted to the class of target analytes.

Table 2 Summary of validation results

Analyte	n=6		Trueness[a] (%)	Precision		Linearity range (ng/mL)	LLOQ (pg/mL)
	Recovery (%)	Matrix effects (%)		Repeatability[a] (%)	IP[a] (%)		
Testosterone	81 %	101 %	98.9–106.8	1.8–6.4	2.2–6.4	0.02–25	20
Epitestosterone	75 %	93 %	95.8–103.3	1.8–7.2	2.5–7.9	0.02–10	20
Androstenedione	77 %	98 %	95.0–105.9	2.1–8.2	2.2–8.2	0.05–25	50
Progesterone	76 %	90 %	99.7–106.8	1.4–5.0	1.4–5.7	0.015–25	15
17α-hydroxyprogesterone	93 %	55 %	97.5–103.5	2.1–6.5	5.4–9.7	0.1–25	100
DHEA	80 %	95 %	98.7–104.6	3.8–9.7	5.7–9.7	0.5–25	500
DHT	78 %	109 %	95.1–103.6	3.1–7.2	3.4–9.4	0.05–10	50
Corticosterone	78 %	113 %	98.3–105.6	3.7–7.0	3.8–7.3	0.1–100	100
Cortisol	82 %	108 %	97.0–105.3	3.3–6.7	3.3–6.7	1–400	1000
Deoxycorticosterone	79 %	93 %	94.2–104.1	1.9–5.4	3.8–9.4	0.025–10	25
11-Deoxycortisol	82 %	99 %	97.0–108.1	1.6–8.2	4.4–9.7	0.025–10	25
Estrone	91 %	94 %	98.6–104.3	2.7–5.1	2.9–5.4	0.005–1	5
Estriol	52 %	115 %	100.5–105.1	3.1–8.9	3.1–8.9	0.005–1	5
Estradiol	97 %	95 %	99.4–103.7	2.3–5.9	2.3–5.9	0.005–1	5

IP: intermediate precision; LLOQ: lower limit of quantification.

[a] See Electronic Supplementary Material for detailed results of each concentration level.

Satisfactory extraction recoveries (n=6) ranging from 75 to 97 % (except for estriol, 52 %) and matrix effects (n=6) from 93 to 115 % (except for 17α-hydroxyprogesterone, 55 %) were obtained at a concentration of 1 ng/mL for all analytes using the approach of Matuszewski et al. [22] (Table 2).

Methods validation

Selectivity and quantitative performances were validated for both developed methods. For the evaluation of selectivity, six depleted serum samples were prepared and analyzed as well as six samples spiked with a steroids mix solution at appropriate low concentration (calibration solution 4, ESM Table S2). The absence of interferences was evaluated on the MRM chromatograms in the region of elution of each steroid, and no significant interfering components were detected. Then, successful and unequivocal identification of all steroids was obtained by comparing chromatographic retention times and relative ion intensities of the two monitored transitions in spiked samples with those of certified steroid solutions (data not shown). Linear calibration curves were generated from the peak area ratio of each steroid to its IS. Selecting a 1/× weighting factor, R^2> 0.99 was obtained for each series for all target steroid hormones. Trueness, repeatability, and intermediate precision were calculated at each concentration level of the validation standards and summary results are shown in Table 2 (all quantitative validation results are presented in ESM Table S3). LLOQ and linearity range of the methods were also determined for each compound (Table 2).

Clinical study samples data treatment

The follow-up of several parameters in a cohort of volunteers generates a data structure naturally forming a three-way tensor (volunteers × parameters × time points). Unfortunately, conventional bilinear methods, such as principal component analysis (PCA) and partial least squares regression (PLS), cannot be applied directly to so-called high-order data without some loss of information [23]. Multiway modeling constitutes a relevant strategy for efficiently extracting biochemical

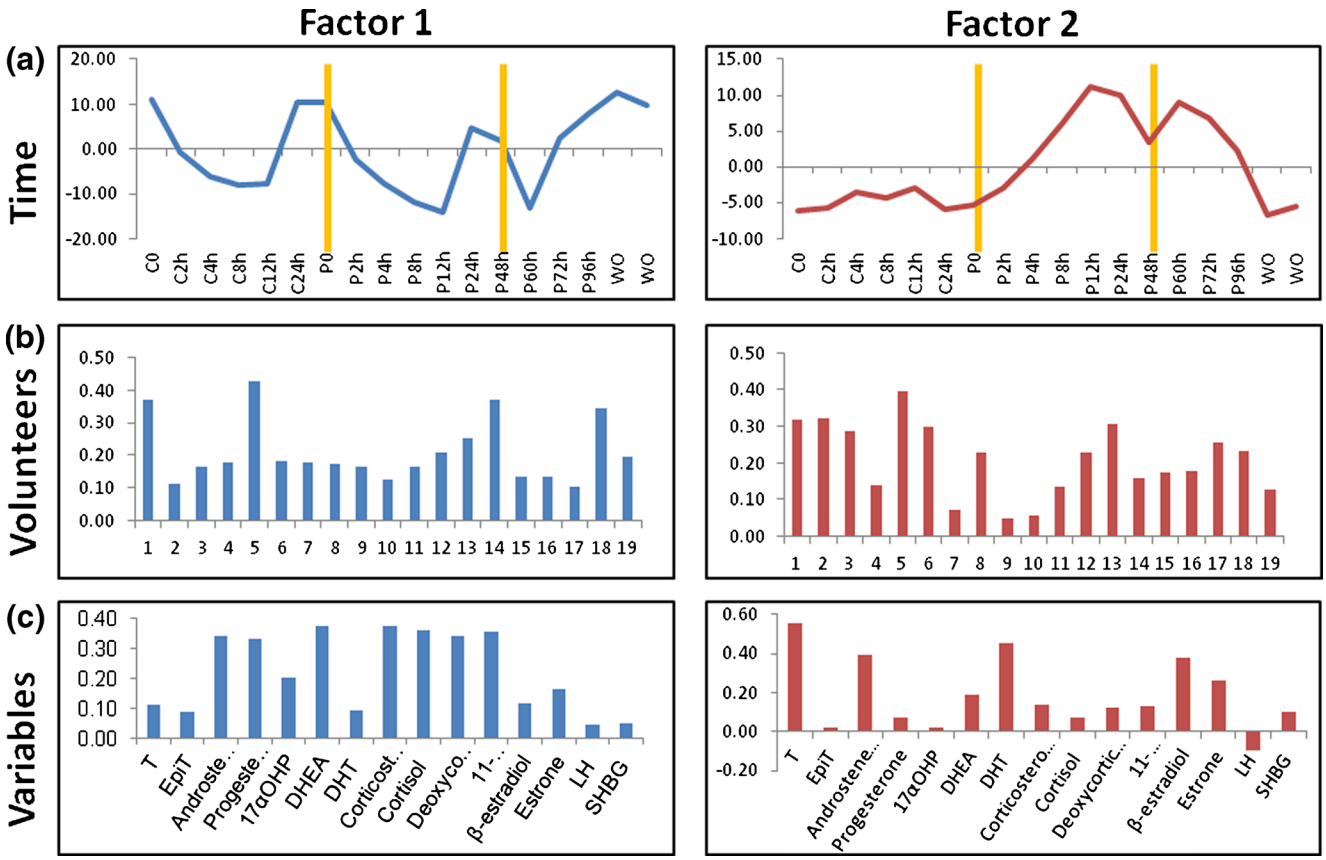

Fig. 2 Score plots of (**a**) time, (**b**) volunteers, and (**c**) variables modes of factors 1 and 2 obtained from PARAFAC modeling on transdermal administration data (50.6 % of explained variance). C: control; P: patch; WO: wash-out

information from this kind of dataset, by preserving relations between the different modes (i.e., variations of specific parameters across time and between volunteers). Unsupervised multiway algorithms include PARAFAC [24] and Tucker models [25]. PARAFAC is a decomposition method adapted to multiway data. In this regard, it constitutes a generalization of PCA to higher order arrays, and the algorithm generates a set of scores and loadings aiming at a compact description of the data structure [26].

Data from transdermal and oral administrations were separately analyzed with the PARAFAC algorithm. Both three-way tensors (time×volunteers×variables) obtained after UHPLC-MS/MS analyses of the control and treatment samples, for transdermal and oral administration respectively, were centered across the time mode while unit variance scaling was applied within the variables mode. Scaling to unit variance within the variable mode is required to assign the same importance to the variability of each of the measured features, regardless of its signal intensity. Data treatment was performed including all compounds except estriol, whose concentrations were in most of the serum samples below the LLOQ.

Transdermal administration

PARAFAC was performed on the 18×19×15 tensor (time×volunteers×variables) and the obtained model was able to capture more than half (50.6 %) of the total variance with only two factors. As presented on Fig. 2, as a major source of variability in the dataset, the circadian rhythm was clearly observed on the first factor (F1) by plotting the score of each time point chronologically. The evaluation of the variables mode allowed androstenedione, progesterone, 17-α-hydroxyprogesterone, DHEA, corticosterone, cortisol, deoxycorticosterone, and 11-deoxycortisol to be highlighted as compounds highly correlated with a circadian modulation. It is to be noted that volunteers showing the most prominent circadian variability (i.e., volunteers 1, 5, 14, and 18) could be detected in the observations mode (Fig. 2b). The second factor (F2) could be related to the transdermal administration of testosterone. An increase was visible on the time scores from 0 to 12 h, followed by a slight decrease until 48 h. The same trend was observed from 48 to 96 h. According to the variables mode, this pattern was mainly associated with altered levels of T, androstenedione, DHT, estradiol and estrone. Looking at

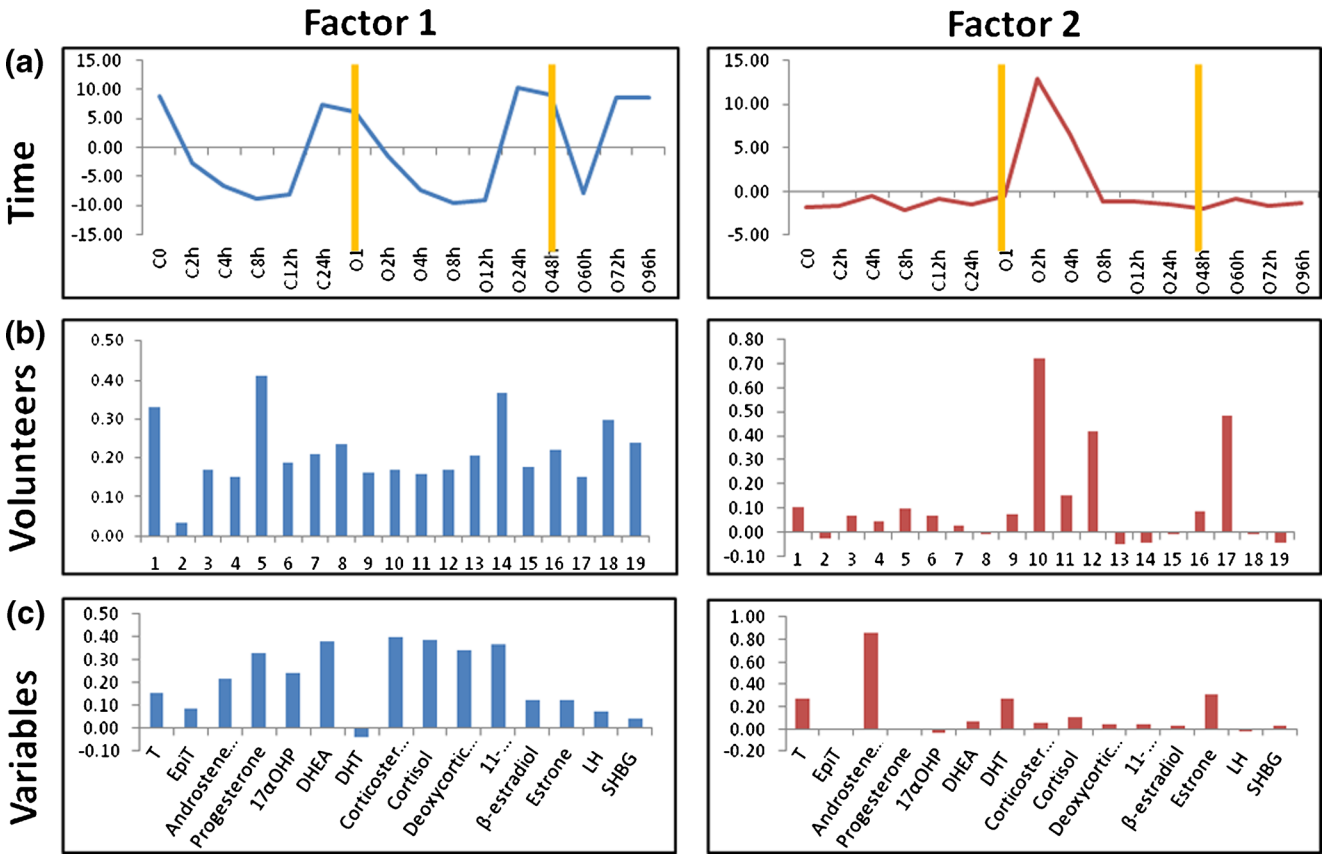

Fig. 3 Score plots of (**a**) time, (**b**) volunteers, and (**c**) variables modes of factors 1 and 2 obtained from PARAFAC modeling on oral administration data (43.3 % of explained variance). C: control; O: oral; WO: wash-out

the observations mode, no significant differences in response to T administration could be noticed between del/del individuals (V5, V12, V14, V15, and V17) and volunteers bearing other genotypes.

Oral administration

PARAFAC was also performed on the 16×19×15 tensor (time×volunteers×variables) and the obtained model explained 43.3 % of the total variance with two factors. Similarly to the transdermal administration, a circadian modulation was first highlighted when assessing the scores of the first factor (F1) in the time mode. Androstenedione, progesterone, 17α-hydroxyprogesterone, DHEA, corticosterone, cortisol, deoxycorticosterone, and 11-deoxycortisol were highly correlated with this pattern, and volunteers 1, 5, 14, and 18 were linked to the highest variations. As previously described, the second factor (F2) was related to the oral administration of testosterone (Fig. 3) and the major biomarkers associated with this trend were androstenedione, T, DHT, and estrone. As seen on the score plot of time mode on F2, the observed biological phenomenon presented an apex at 2 h with a rapid decrease until 8 h. Because of this limited detection window, changes related to the second oral administration could not be detected when analyzing time points after 48 and 60 h. It has to be noted that volunteers 10, 12, and 17 showed more markedly altered steroid profiles due to oral administration than others but, as for transdermal administration, no significant differences between the three genotypes were noticed.

Circadian rhythm

The PARAFAC models highlighted variables following a circadian rhythm and/or highly related to testosterone administration. This information is of high importance to select relevant parameters for further longitudinal monitoring. The circadian rhythm was therefore studied in detail during the control phase for all subjects (24-h period). Mean serum concentration values of testosterone and cortisol at each collection time point are presented for all volunteers in Fig. 4 (graphs of all other investigated compound are in ESM Fig. S1). As expected, it was observed that most of the investigated hormones were drastically influenced by the circadian rhythm. This included mainly eight hormones (androstenedione, progesterone, 17α-hydroxyprogesterone, DHEA, corticosterone, cortisol deoxycorticosterone, and 11-deoxycortisol), which were highly secreted in blood in the morning and concentration of which decreased along the day. For the other compounds (testosterone, epitestosterone, DHT, LH, estrogens, and SHBG) the circadian variation was less pronounced. These trends were in accordance with the results of PARAFAC modeling, which highlighted the same eight hormones as the most affected by circadian rhythm (F1, Figs. 2 and 3).

In doping control, the intra-day fluctuations of these compounds could therefore constitute an important limitation in their use as markers of steroid abuse in a longitudinal approach; indeed, circadian rhythm masks all possible smaller variations that could be related to T administration. For this reason, substances showing a pronounced circadian rhythm were discarded for a first tentative longitudinal monitoring (see below), but further investigation should be carried out in the future.

Longitudinal monitoring of biomarkers

As a result of PARAFAC modeling (Factor 2), five steroid hormones were highlighted as possible markers for the detection of T misuse: testosterone, DHT, androstenedione, estradiol, and estrone. The usefulness of each marker for detecting T misuse was evaluated by setting subject-based thresholds.

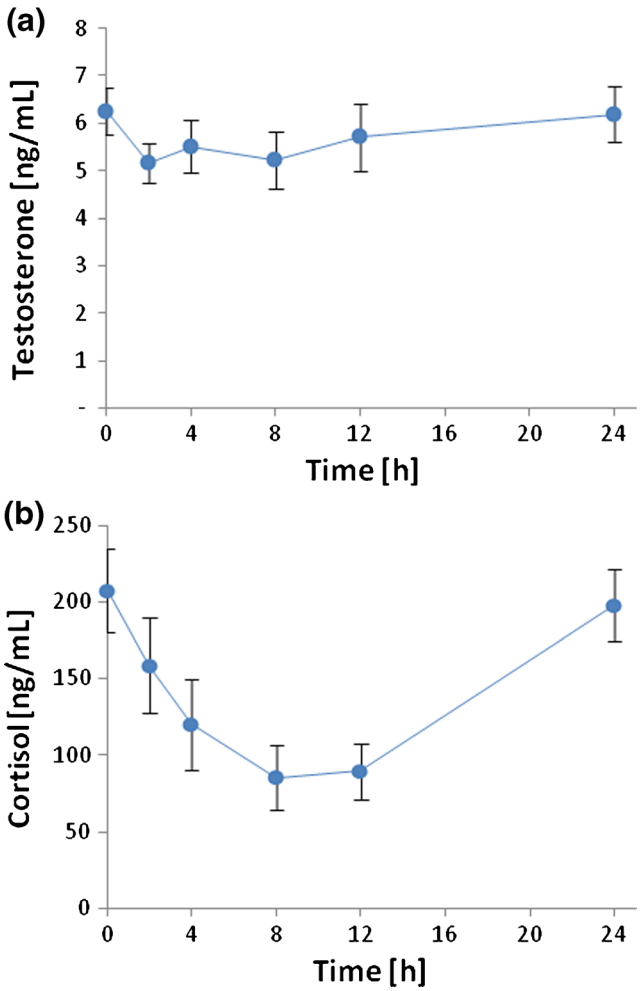

Fig. 4 Serum concentrations and circadian rhythm of (**a**) testosterone and (**b**) cortisol over 24 h during control period expressed with CI at 95 %

For this purpose, the six data points collected during the control wk (C0 to C05) were used to define individual thresholds for each volunteer by using the formula [mean±3×SD], as already reported in literature [27]. Detection time was defined as the time after T administration when the measured concentration was higher than the individual threshold, assuming that T administration led to increased levels of markers.

As they were considered the most promising biomarkers, longitudinal monitoring of T, DHT, and androstenedione was performed for each volunteer and both administration routes. On the other hand, estradiol and estrone profiles could not be clearly linked to T administration (data not shown). The high variability observed for the latter compounds could be explained by instrumental causes due to low concentration of estrogens measured in male serum samples, and they were therefore not further considered in the present study.

Transdermal administration

Longitudinal follow-up of T, DHT, and androstenedione was performed and significant augmentations of T and DHT concentration were noted on the profiles. For androstenedione, no evident variation in serum concentrations was observed after T transdermal administration.

Detection windows of T and DHT for all volunteers are summarized in Fig. 5a. Both T and DHT showed considerable detection capability based on individual thresholds, with their concentrations rising beyond these limits for all volunteers. Testosterone concentrations higher than individual threshold were found for 60.2 % of the samples collected after T administration, with detection windows between 2 and 96 h, considering also the second T patch application. Interestingly, the percentage of "positive" samples for DHT was significantly higher (71.3 %), with the same relevant detection window. A rapid conversion of T into DHT during its absorption via 5α-reductase pathway may be responsible for this observation [9, 28]. It is also important to note that no difference was observed in T and DHT response for the three genotypes; in particular, serum concentrations of both markers easily reached individual limits for all del/del volunteers, as shown in Fig. 5a. This represents a major improvement compared with results of the "urinary steroid profile," for which the detection of T misuse in people bearing del/del genotype is known to be problematical [13] and certainly less sensitive than the approach proposed herein. An example of serum concentration profiles for one volunteer of each genotype is shown in Fig. 6a for T and 6b for DHT, respectively. In addition, the urinary T/E ratios of the same three volunteers are also shown in Fig. 6c for comparison

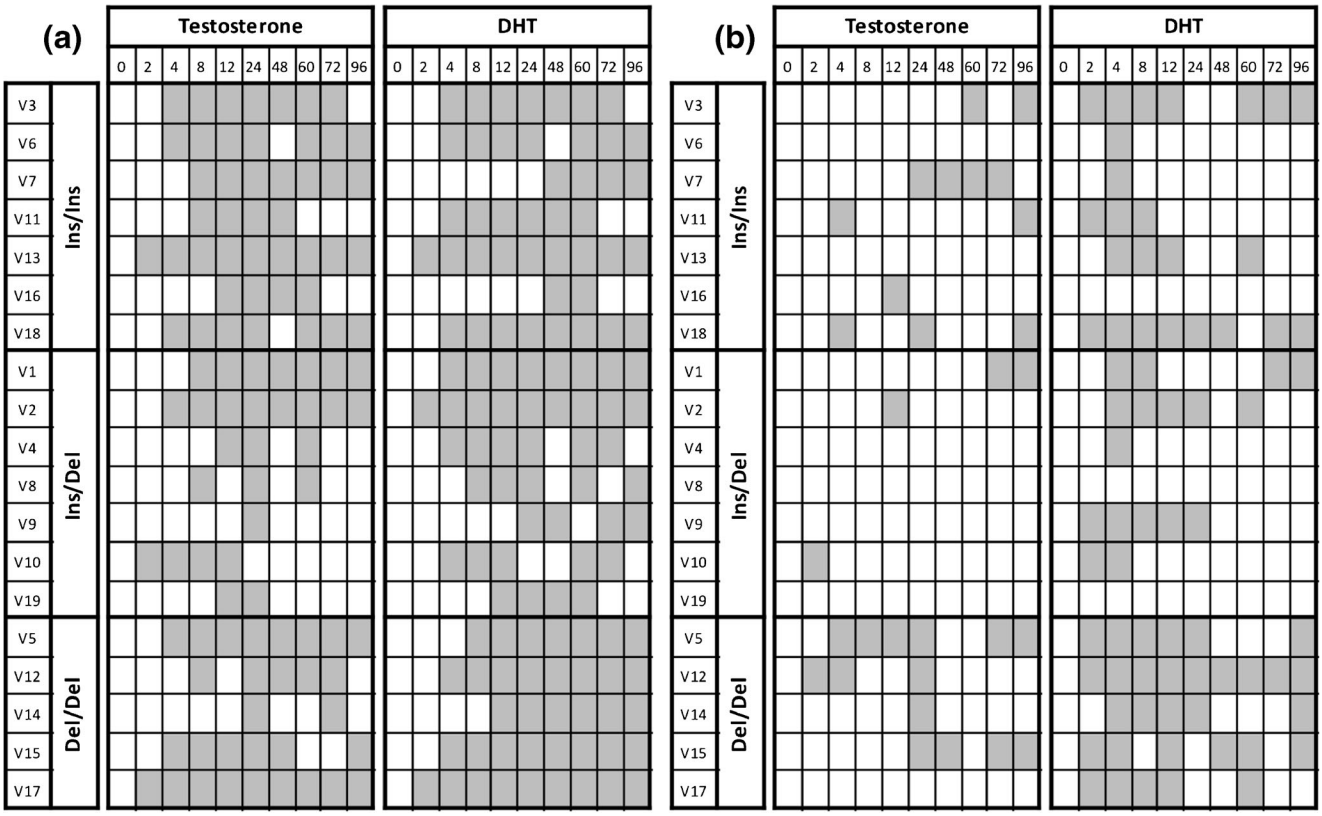

Fig. 5 Testosterone and DHT detection times for (**a**) transdermal T administration and (**b**) oral TU administration (grey-filled box=concentration higher than individual threshold)

purposes. These data were previously obtained by GC-MS quantification of urines of the same clinical study [12]. From these graphs, it can be highlighted that both T and DHT concentrations in serum increased from 2 to 4 times after patch administration in comparison with values obtained during the control period. For all three volunteers, T and DHT individual thresholds are exceeded after 2 to 4 h from application and stay outside the limits until 96 h. The second application does not result in a raise of T and DHT concentrations higher than after the first application. This observation could be due to a lower T release by passive diffusion through the skin.

As already reported for urinary data [12], the T/E never reached the individual threshold for volunteer 17 (del/del), while for volunteer 2 (ins/del) a four times T/E increase was observed only after the second patch application (see Fig. 6c).

Oral administration

Longitudinal monitoring of T, DHT and androstenedione was also performed for oral administration samples. As for the transdermal application, T and DHT variations could be linked to testosterone administration, but the ability of detecting TU oral misuse is manifestly less pronounced compared with the one observed for transdermal application. Detection times of these two compounds for all volunteers are summarized Fig. 5b. DHT showed moderate detection capability based on individual thresholds, with its serum concentration rising beyond individual limits for 16 out of 19 volunteers. DHT concentrations higher than individual threshold were found for 42.1 % of the samples collected after TU administration, with detection windows prevalently between 2 and 12 h, considering the first TU intake. Maximum concentrations were generally achieved 2 h after the administration, with a 2- to 3-fold increase of serum concentration values in comparison with control samples. On the other hand, T did not show the same detection performance, with concentration values that never reached individual thresholds for 6 out of 19 volunteers and a percentage of "positive" samples of only 17.5 %. An example of serum concentration profiles for one volunteer of each genotype is shown in Fig. 7a for T and 7b for DHT and compared with urinary T/E (Fig. 7c). From these results, the urinary T/E had a much clearer response for volunteer 13 (ins/

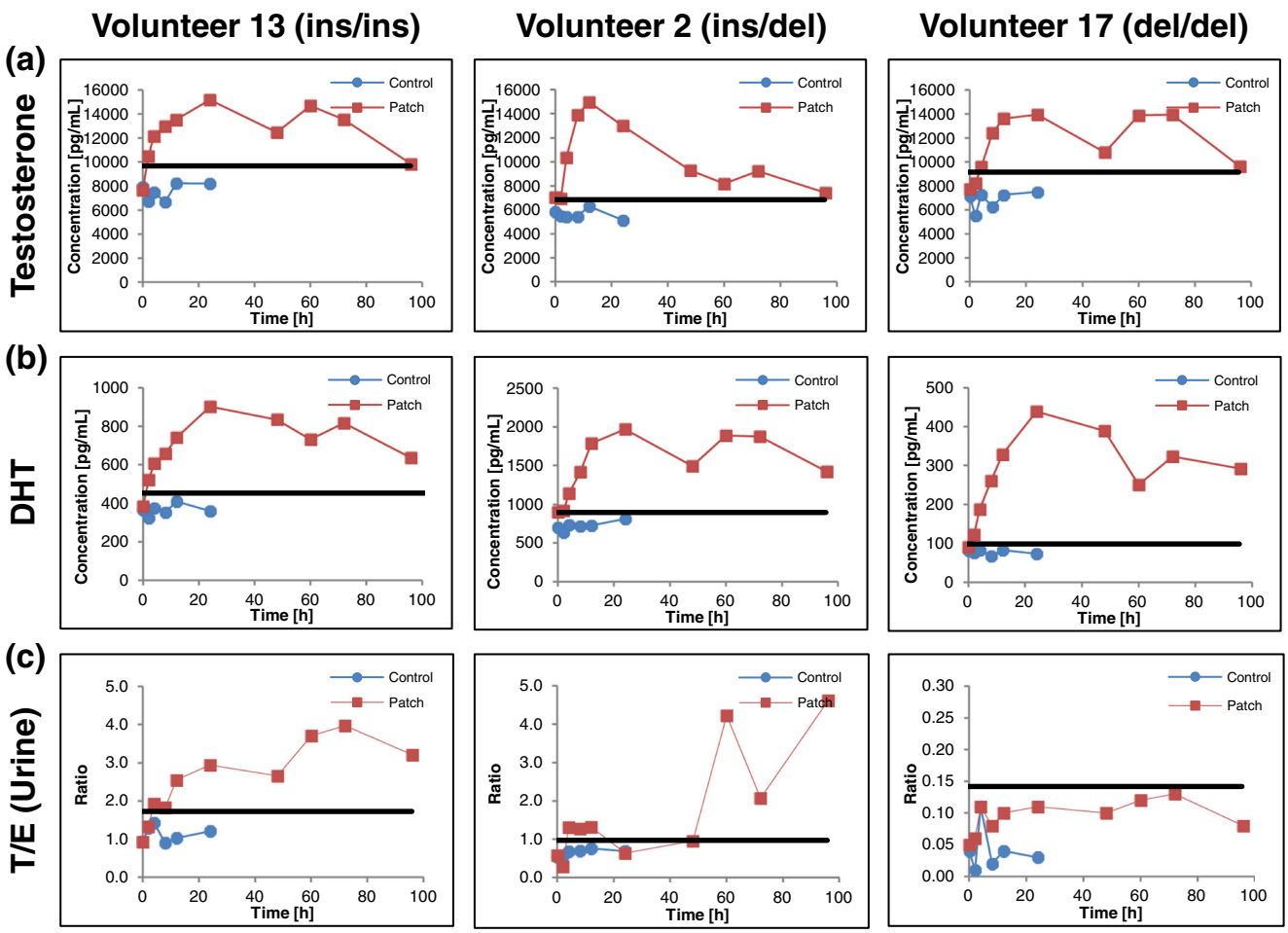

Fig. 6 Longitudinal monitoring of (**a**) T and (**b**) DHT serum concentrations after transdermal T administration and (**c**) their comparison with T/E urinary ratio [12]

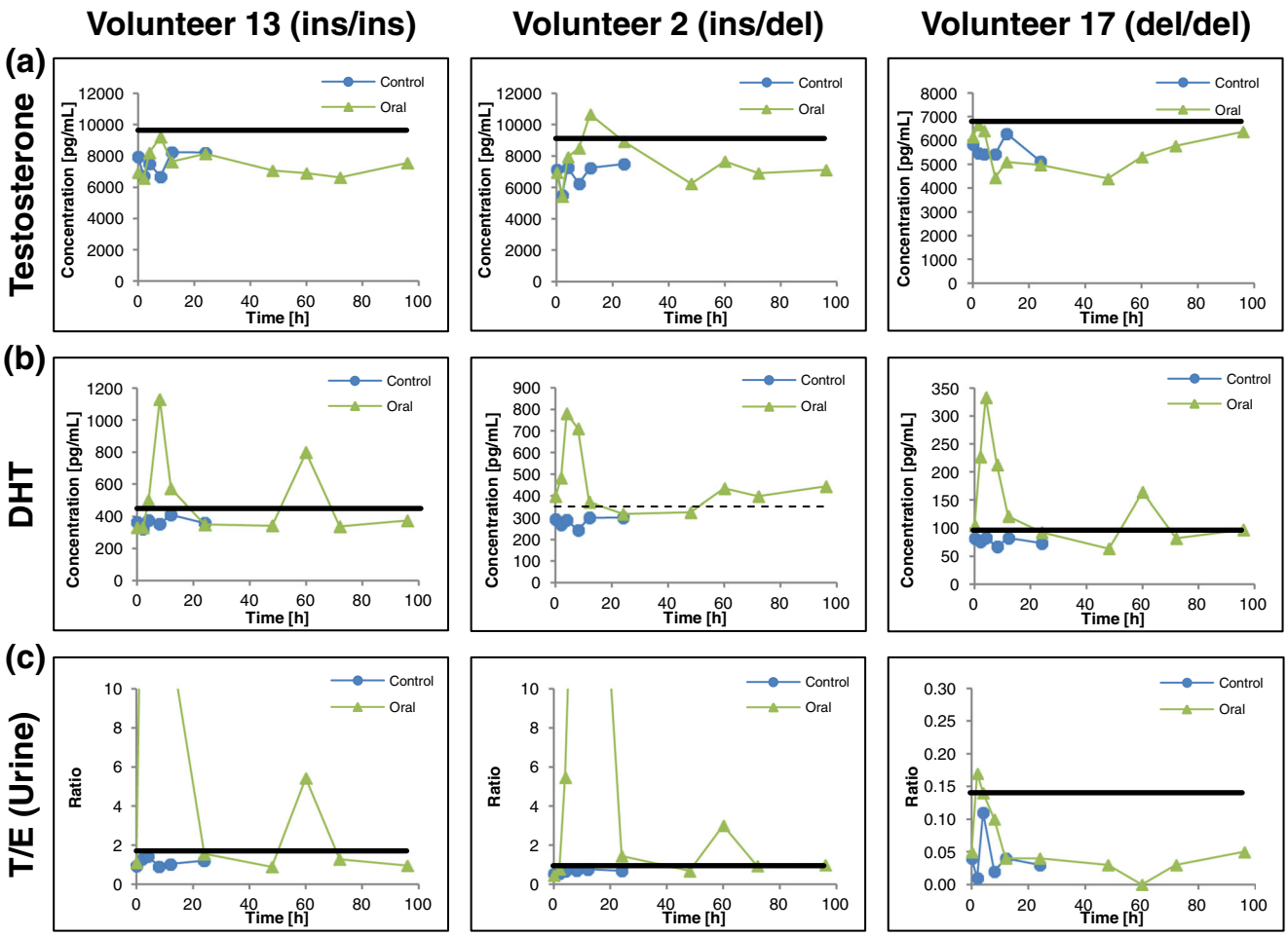

Fig. 7 Longitudinal monitoring of (**a**) T and (**b**) DHT serum concentrations after oral TU administration and (**c**) their comparison with T/E urinary ratio [12]

ins) and volunteer 2 (ins/del) compared with serum T and DHT, which did not show any improvements in terms of detection windows, whereas in the case of volunteer 17 (del/del), for which urinary markers are less sensitive, DHT serum concentration provided very encouraging results.

In addition, as observed from PARAFAC analysis (see "Oral administration" section), androstenedione was also highlighted as a promising marker of TU administration. An acute hormonal response related to this compound was observed for 4 out of 19 volunteers, with a maximum concentration observed 2 h after TU intake (ESM Fig. S2). This observation could be explained by a T retroconversion to androstenedione as already reported after 50 mg oral DHEA or 20 mg androstenedione sublingual ciclodextrin tablet in young males, which are both precursor of T biosynthesis [29, 30]. This acute response, however, could not be attributed to a particular genotype as it occurred for two individuals bearing the del/del genotype, for one ins/ins, and one ins/del.

The results obtained after oral administration highlighted the limitations associated with the targeted analysis of a reduced set of known parameters. In this context, untargeted analyses constitute a promising way to improve the detection of forbidden substance misuse by extending the range of measured compounds. A metabolomic approach could reveal additional and more sensitive biomarkers for the detection of T misuse via oral administration route. This will be the subject of future work.

Conclusion

The shift from population-based limits to the longitudinal follow-up of individual athletes represents an ongoing trend in anti-doping controls since the introduction of Athlete Biological Passport in 2009. In this research, serum samples from a clinical study of transdermal and oral T administration were analyzed. We applied the longitudinal monitoring to serum concentrations of T and DHT, which were highlighted by unsupervised multiway modeling (PARAFAC) as the most promising biomarkers of T misuse among the 13 steroid hormones quantified by validated UHPLC-MS/MS methods. Results showed that after T administration, serum concentrations of T (oral) and DHT (oral and transdermal) exceeded

individual thresholds for most of the volunteers. This first example of blood "steroid profile" confirms the hypothesis that longitudinal follow-up of steroid hormones in serum could be a valid complement to urinary "steroid profile" used currently, in particular for cases involving del/del individuals and for transdermal application, which is probably the administration route preferred by athletes today. Moreover, for del/del individuals the measurement of urinary T/E is known to be particularly critical because natural concentration values of testosterone glucuronide are 10–50 times lower than other genotypes, near or even below the LOD of state of the art GC-MS(/MS) methods, generating suspect "steroid profiles" that could be related to analytical issues in T and E quantification at low concentration levels. This limitation was overcome by the analysis of the blood matrix, for which no difference between UGT2B17 genotypes were observed.

In order to possibly implement longitudinal monitoring of serum concentrations in routine anti-doping controls, supplementary studies should be carried out with the aim of evaluating the presence of eventual confounding factors in this matrix (such as the influence of alcohol or the use of masking agents) and the possible influence of other polymorphisms, such as CYP17. However, results of the presented study demonstrate that blood could be a suitable matrix for longitudinal monitoring in the context of T misuse. This strategy could also be applied in perspective to other class of endogenous hormones in view of a future implementation of an "endocrinological module" in the Athlete Biological Passport.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

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