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Federico Ponzetto[a*], Mirko Parasiliti-Caprino [a], Iacopo Gesmundo [a], Lorenzo Marinelli [a], Antonello Nonnato [b], Raul Nicoli [c], Tiia Kuuranne [c], Giulio Mengozzi [b], Ezio Ghigo [a], Fabio Settanni. [a b]

[a]Division of Endocrinology, Diabetes and Metabolism, Department of Medical Sciences, University of Turin; Turin, Italy
[b]Clinical Biochemistry Laboratory; City of Health and Science University Hospital; Turin, Italy
[c]Swiss Laboratory for Doping Analyses, University Center of Legal Medicine Geneva and Lausanne, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Switzerland

Corresponding author. Division of Endocrinology, Diabetes and Metabolism Department of Medical Sciences; University of Turin Corso Dogliotti 14, 10126 Turin, Italy E-mail address: federico.ponzetto@unito.it (F. Ponzetto)

ABSTRACT

Anti-doping rule violations related to the abuse of endogenous anabolic androgenic steroids can be currently discovered by the urinary steroidal module of Athlete Biological Passport. Since this powerful tool is still subjected to some limitations due to various confounding factors altering the steroid profile, alternative strategies have been constantly proposed. Among these, the measurement of blood concentrations of endogenous steroid hormones by LC-MS is currently of increasing interest in anti-doping, bringing significant advantages for the detection of testosterone abuse in females and in individuals with deletion of UGT2B17 enzyme. Although various research groups have made significant efforts in method development, there is currently no accepted or harmonized anti-doping method for quantitative analysis of the various testosterone doping markers in blood. In this study we present a UHPLC-MS/MS method for the quantification of major circulating steroid hormones together with an extended panel of glucuro- and sulpho-conjugated phase II metabolites of androgens. Chromatographic setup was optimized by comparing the performance of three different C18 stationary phases and by the careful selection of mobile phases with the aim of separating all the target steroids, including numerous isomeric/isobaric compounds. MS parameters were fine-tuned to obtain the sensitivity needed for measuring the target analytes, that show specific serum concentrations ranging from low pg/mL for less abundant compounds to µg/mL for sulpho-conjugated steroids. Finally, sample preparation protocol was developed for the extraction of steroid hormones from 200 µL of serum and the performance was evaluated in terms of extraction recovery and matrix effect. The final method was then applied to authentic serum samples collected from healthy volunteers (40 males and 40 females) at the Blood Bank of the City of Health

and Science University Hospital of Turin. The analysis of these samples allowed to obtain results on serum concentrations of the targeted steroids, with particular emphasis on previously undiscovered phase II metabolites, such as the isomers of 5-androstane-3,17-diol glucuronide. This preliminary application also enabled measuring dihydrotestosterone sulphate in male samples, efficiently separating this analyte from its isomer, epiandrosterone sulphate, which circulates in blood at high concentrations. The promising results of this study are encouraging for the measurement of blood steroid profile markers in serum and plasma samples for Athlete Biological Passport purposes.

Highlights

- Lack of comprehensive analytical method for blood steroid profiling
- UHPLC-MS/MS method for quantification of 27 steroids and conjugated metabolites
- Optimized separation of isomers and efficient extraction of selected steroids
- Quantitative validation of the developed method following ISO and WADA guidelines
- Reference intervals of steroid hormones and metabolites in healthy population

Keywords – Steroids; Serum; Doping; UHPLC-MS/MS; Androgens
Athlete biological passport.

Abbreviations

21-DFn - 21-Deoxycortisol
5ααβ-Adiol 17-G - 5α-androstan-3α,17β-diol 17-glucuronide
5ααβ-Adiol 3-G - 5α-androstan-3α,17β-diol 3-glucuronide
5αββ-Adiol 17-S - 5α-androstan-3β,17β-diol 17-sulphate
5αββ-Adiol 3-G - 5α-androstan-3β,17β-diol 3-glucuronide
5βαβ-Adiol 17-G - 5β-androstan-3α,17β-diol 17-glucuronide
5βαβ-Adiol 3-G - 5β-androstan-3α,17β-diol 3-glucuronide
A-S - Androsterone sulphate
ABP - Athlete Biological Passport
ACN - Acetonitrile
BSP - Blood Steroid Profile
DHEA-S - Dehydroepiandrosterone sulphate
DHT - Dihydrotestosterone
DHT-S - Dihydrotestosterone sulphate
DOC - 11-Deoxycorticosterone
E-G - Epitestosterone glucuronide
EAAS - Endogenous Anabolic Androgenic Steroids
EpiA-S - Epiandrosterone sulphate
Etio-S - Etiocholanolone sulphate
H_3PO_4 - Phosphoric acid
IQR - Interquartile range
IRMS - Isotopic ratio mass spectrometry

IS - Internal standard
LLOQ - Lower limit of quantification
MeOH - Methanol
NH_4OH - Ammonium hydroxide
P - Progesterone
QC - Quality control
QCF - Female serum samples
QCM - Male serum samples
SPE - Solid phase extraction
T - Testosterone
T-G - Testosterone glucuronide
T-S - Testosterone sulphate
WADA - World Anti-Doping Agency

1. INTRODUCTION

Athlete Biological Passport (ABP), which was initially implemented in doping control analyses with the hematological module, was enhanced in 2014 by the addition of the steroidal module, with the aim of improving the detection of doping practices with endogenous anabolic androgenic steroids (EAAS) [1]. This module consists of the longitudinal monitoring of five ratios between urinary concentrations of androgens, which are measured by (GC-MS(/MS)), and for which the individual limits are calculated by a Bayesian adaptive model. The monitored ratios, known to be altered by the administration of synthetic forms of EAAS, are: androsterone/testosterone, androsterone/etiocholanolone, 5α-androstane-3α,17β-diol/5β-androstane-3α,17β-diol, 5α-androstane-3α,17β-diol/epitestosterone and the most significant, testosterone/epitestosterone (T/E) [2]. This longitudinal approach, despite having significantly improved the detection capabilities of doping with T compared to the previous threshold value of the T/E ratio set at 4 [3,4], still presents some drawbacks mainly related to the characteristics of urinary matrix and to the analytical technique employed. Indeed, the GC-MS(/MS) analytical procedure employed routinely in the laboratories accredited by World Anti-Doping Agency (WADA) could in some cases suffer from a lack of sensitivity in measuring T/E ratio in individuals bearing the del/del genotype for UGT2B17 polymorphism [5,6]. Furthermore, such analytical procedure requires a hydrolysis step that, cleaving the chemical bond with the glucuronic substituent, results in the measurement of total steroid concentration (free + glucuro-conjugated) and hence reduces important information regarding phase II metabolism. In addition to this, it has already been demonstrated that the results and data interpretation from quantitative urinary steroid analysis in urine can be strongly influenced by the presence of both endogenous (e.g., metabolism, ethnicity) and exogenous (e.g., bacterial contamination, alcohol consumption) confounding factors and especially genetic enzymatic deficiencies (e.g., deletion of UGT2B17 enzyme) [[7], [8], [9], [10]]. Recently, the efforts of the anti-doping community have focused on discovering and evaluating different analytical approaches for the detection of EAAS misuse: besides the research of novel urinary markers capable of extending doping detection windows as well as increasing sensitivity [[11], [12], [13], [14]], the measurement of endogenous circulating steroids concentrations is rapidly taking hold within the anti-doping context and nowadays represents the last frontier of the research on steroid profiling. In fact, the blood matrix ensures a representative and reliable sample collection and can provide a snapshot of the physiological status of an individual. Furthermore, the longitudinal monitoring of endogenous steroids concentrations in blood could furnish more accurate information about androgen

metabolism, which could be of additional value in the interpretation of individual steroid profiles [[15], [16], [17]].
In previous studies it has been proven that the longitudinal monitoring of T and dihydrotestosterone (DHT) serum concentrations could bring complementary information to the currently employed urinary steroidal module, by eliminating the impact of UGT2B17 polymorphism and by increasing the detection capability of transdermal application of T [18]. Following these findings, in 2019 the first two cases of elite female athletes sanctioned for T doping based on the measurement of atypical circulating T levels in serum were reported [17]. Welcoming this significant decision by the Court of Arbitration for Sport, WADA also stated that "the measurement of testosterone levels in blood serum constitutes a further tool […] to detect doping", officially opening the way for the implementation of such approach in anti-doping context. Furthermore, thanks to a steroidomic study, additional markers were highlighted among the phase II metabolites of androgens (glucuro- and sulpho-conjugates), demonstrated to be more sensitive and capable of extending the detection window of doping with oral T [19]. More recently, the research on blood steroid profiling significantly increased, with the research groups of WADA-accredited laboratories focusing their work on specific objectives, such as evaluating the performance of Blood Steroid Profile (BSP) in female populations and comparing blood markers to the currently used urinary ones [[20], [21], [22]]; developing novel analytical methods for measuring reported markers in serum matrix as well as in alternative matrices (e.g., dried blood spots) [23,24]; testing novel approaches for detecting EAAS doping in blood matrix, such as the application of linear discriminant models [25] and expanding isotopic ratio mass spectrometry (IRMS) confirmatory analysis in blood [26].
Despite the efforts made by the scientific anti-doping community, there is still lack of approved and harmonized method for the quantitative analysis of all the highlighted EAAS doping markers. Indeed, the majority of reported LC-MS/MS method for the measurement of T doping markers in serum limit their monitoring to endogenous free steroids, focusing their attention to T, DHT and androstenedione [18,20,22,25]. While the extended steroid profiling approaches recently employed to expand the knowledge regarding androgens response to T administration were not able to efficiently detect and separate all circulating phase II metabolites that proved to be influenced by EAAS doping [19,21,24]. The aim of this research was to develop and validate a rapid and comprehensive UHPLC-MS/MS method for the simultaneous measurement of a panel of up to 27 endogenous steroids in serum, including both the most important circulating steroid hormones and the various phase II androgen metabolites. The method was then applied to the investigation of circulating levels of target steroidal compounds in a healthy population of 40 males and 40 females, providing a first insight of androgen metabolism.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Steroid certified reference materials and labelled internal standards (IS) were purchased from the following providers. 11-Deoxycorticosterone (DOC), 11-Deoxycortisol, 17α- Hydroxyprogesterone, 21-Deoxycortisol (21-DF), 5α-androstan-3α,17β-diol 17- glucuronide (5ααβ-Adiol 17-G), 5α-androstan-3β,17β-diol 17-sulphate (5αββ-Adiol 17-S), 5α- androstan-3α,17β-diol 3-glucuronide (5ααβ-Adiol 3-G), Androstenedione, Androsterone glucuronide, Androsterone sulphate (A-S), Corticosterone, Cortisol, Cortisone, Dehydroepiandrosterone, Dehydroepiandrosterone sulphate (DHEA-S), DHT, Epiandrosterone sulphate (EpiA-S), Epitestosterone (E), Epitestosterone glucuronide (E-G), Epitestosterone sulphate, Etiocholanolone glucuronide, Etiocholanolone sulphate (Etio-S), Progesterone (P), T, Testosterone glucuronide (T-G), Testosterone sulphate (T-S) as well as 11- Deoxycorticosterone D8, 17a-Hydroxyprogesterone D8, Androstenedione D7, Corticosterone D8, DHT D3, Epitestosterone D5, Progesterone D9, Testosterone D3 were provided by Steraloids (Newport, RI, USA); 5β-androstan-3α,17β-diol 3-glucuronide (5βαβ-Adiol 3-G), 5α-androstan-3β,17β-diol 3-glucuronide

(5αββ-Adiol 3-G), 5β-androstan-3α,17β-diol 17-glucuronide (5βαβ-Adiol 17-G), Dihydrotestosterone sulphate (DHT-S) as well as 5α-androstan-3α,17β-diol 17-glucuronide D4, 5β-androstan-3α,17β-diol 17-glucuronide D3, Androsterone sulphate D4, Cortisol D4, Cortisone D8, Epitestosterone sulphate D3, Etiocholanolone glucuronide D5, Etiocholanolone sulphate D5, Testosterone glucuronide D3, Testosterone sulphate D3 were obtained from LGC Standards (Teddington, United Kingdom); 11-Deoxycortisol D5, 21-Deoxycortisol D8, DHEA D5, DHEA-S D5, Androsterone glucuronide D4 were purchased from Merck KGaA (Darmstadt, Germany).
UHPLC-MS grade methanol (MeOH) and acetonitrile (ACN) were purchased from Carlo Erba Reagents S. r.l. (Cornaredo, Italy), while ammonium acetate, ammonium formate, ammonium hydroxide solution (NH_4OH, 28–30%) and phosphoric acid solution (H_3PO_4, ≥85%) were obtained from Merck KGaA (Darmstadt, Germany). Deionized water was obtained by a Milli-Q®-grade system (Millipore, USA) and was used for the preparation of all LC mobile phases and aqueous solutions. Charcoal Dextran Stripped Human Serum was acquired from Innovative Research Inc. (Novi, MI, USA).
For each analyte and IS, stock solutions were prepared at a concentration of 1 mg/mL (2 mg/mL for A-S, DHEA-S, EpiA-S and Etio-S) in MeOH and stored in 2 mL amber glass vials at −20 °C; intermediate solutions at appropriate concentrations (1 mg/mL, 100 µg/mL, 10 µg/mL, 1 µg/mL, 100 ng/mL, 10 ng/mL) were prepared through consecutive dilution of the stock solutions in MeOH and stored in 10 mL glass tubes at −20 °C. Working solutions containing all target analytes were prepared in MeOH and for the preparation of the different calibration and validation samples (concentration details in Supplementary Material Table S1) 20 µL of the appropriate working solution were spiked in depleted serum. A solution containing all IS (IS-mix) was prepared spiking different volumes of each IS intermediate solution at appropriate concentration for each IS in MeOH (concentration details in Supplementary Material Table S2) and stored in 10 mL glass tubes at −20 °C. Stability of stored analytes' and IS solution was evaluated, together with instrument performance, by injecting each day of analysis a system suitability test solution containing all analytes and IS at fixed concentration and by monitoring detected peaks' areas. The concentration of each IS in the IS-mix was selected by evaluating the lowest concentration of IS that could be detected in samples with a satisfactory repeatability without causing interference to or being altered by relative analyte signal.

2.2. Sample preparation

Solid phase extraction (SPE) was employed for extracting target steroid hormones from serum samples using EVOLUTE® EXPRESS ABN 30 mg (Biotage, Uppsala, Sweden) 96-well plates and applying positive pressure with Resolvex® A200 automated processor (Tecan, Männedorf, Switzerland). Prior to sample loading, each well was conditioned with 1 mL of MeOH and then equilibrated with 1 mL of 2% H_3PO_4 solution, applying after each step a high positive pressure gradient of 1 min. Serum sample pre-treatment was carried out by spiking 25 µL of the IS- mix in a sample aliquot of 200 µL, diluting the latter with 200 µL 2%H_3PO_4 and then agitating the sample for 15 min at 600 rpm. Each well was then loaded with 425 µL of pre-treated serum sample and a high positive pressure gradient of 1 min was applied. Elution was then performed adding 700 µL of a MeOH/ACN 1/1 (v/v) solution to each well and applying a low positive pressure gradient of 3 min. The extracts were collected in collection plates equipped with 1.5 mL glass inserts, evaporated for approximately 30 min at 50 °C under a stream of nitrogen and finally reconstituted with 100 µL of a MeOH/H_2O 1/1 (v/v) solution. The plate was then gently shaken for 15 min at 300 rpm and 20 µL of each extract were finally injected into UHPLC-MS/MS for analyses. A schematic representation of the whole sample preparation workflow is presented in Fig. 1.

2.3. UHPLC-MS/MS analysis

Analyses were carried out using a Nexera X2 UHPLC system (Shimadzu, Tokyo, Japan) coupled to a Citrine Triple Quad MS/MS system (AB Sciex, Ontario, Canada). The system control and data analysis were performed by AB Sciex Analyst and MultiQuant software, respectively. Chromatographic

separation was achieved using Kinetex PS C18 column (150 × 2.1 mm, 2.6 µm; Phenomenex, Torrance, USA) set at 55 °C. Mobile phase A was 10 mM ammonium acetate in H_2O and mobile phase B was 10 mM ammonium acetate in MeOH. The gradient started linearly from 10% to 50% B over 1 min, followed by an isocratic step at 50% B for 2.5 min, a further increase to 60% B over 5 min and a second isocratic step at 60% B for 2.5 min; the gradient continued with a last increase from 60% to 80% B over 4 min followed by a washing step at 98% B for 6 min and finally the column was re-equilibrated for 4 min at initial conditions for a total run time of 25 min. The injected volume was 20 µL and the flow rate was set at 300 µL/min.
ESI-MS/MS analysis was performed in polarity switching mode. Source temperature was maintained at 550 °C and Ion spray voltage was set at 5500 and -4500 V in positive and negative ionization modes, respectively. Curtain gas pressure was set at 35 psi, nebulizer gas pressure at 45 psi and heater gas pressure at 60 psi. Two MS/MS transitions (quantifier, qualifier) were selected for each target analyte, except for sulphated steroids detected in negative ionization mode for which only one fragment was detected, while one MS/MS transition was selected for IS. Declustering Potential, entrance potential, collision energy and cell exit potential for each MS/MS transition were optimized by infusing standard solutions of each target analyte and relative IS at 100 ng/mL in reconstitution solvent (MS parameters details in Supplementary Material Table S3).

2.4. Method validation

The method was validated following the ISO/IEC 17025:2017 requirements [27] that represent a mandatory standard for WADA-accredited laboratories performing doping control analyses. A validation protocol that included the assessment of selectivity, matrix effects, extraction recoveries, quantitative performance (trueness, repeatability, intermediate precision, combined uncertainty, linearity range, lower limit of quantification (LLOQ)), carry-over, robustness as well as stability of extracts and calibration curves was set up. Selectivity was evaluated by means of extracting and analyzing five depleted serum samples (negative control), five depleted serum samples spiked with the Level 4 calibration solution, five real female serum samples and five real male serum samples, comparing fragments ions' ratios measured in spiked samples with the ones measured in male and female serum samples. Furthermore, five depleted serum samples spiked with a solution containing approximately 60 exogenous steroids at concentration between 2 and 5 ng/mL were also extracted and analyzed with the aim of evaluating the presence of chromatographic interferences in the selected MRM transitions of all target analytes due to such structurally similar compounds. For each of the 27 target analytes, extraction recoveries and matrix effects were measured employing the approach of Matuszewski et al. [28]. Extraction recoveries were calculated as the ratio between peak areas of depleted serum samples spiked before and after the extraction protocol with a mix of target analytes. Matrix effects were investigated by comparing the peak area of depleted serum samples spiked after extraction with that of the corresponding methanolic standard solution containing all the target analytes. For these experiments, the Level 4 calibration sample was employed (Supplementary Material Table S1). Furthermore, the impact of hemolysis was evaluated by performing two different experiments: a firs one extracting serum samples with different degrees of hemolysis measured by photometric tests (1 with hemoglobin (Hgb) concentration <50 mg/dL, 2 with [Hgb] between 50 and 99 mg/dL, 2 with [Hgb] between 100 and 199 mg/dL and 2 with [Hgb] between 200 and 299 mg/dL) and evaluating the possible signal suppression or enhancement of spiked IS-mix; a second one creating three serum pools with different degrees of hemolysis ([Hgb] 50–99 mg/dL; [Hgb] 100–199 mg/dL; [Hgb] 200–299 mg/dL) and spiking them as well as charcoal dextran stripped serum with Level 5 calibration solution and evaluating the presence of chromatographic interferences in the region of elution of all target analytes in the selected MRM transitions. Back conversion of IS deuterated compound was also assessed by injecting IS-mix diluted 1:5 in MeOH/H2O 1/1 (v/v) solution and by evaluating the presence of interference in relative analytes' MRM transitions. Quantitative performance of the method (trueness, repeatability, intermediate precision, combined uncertainty, linearity range, LLOQ) were assessed on three

different analytical series performed on three days by two operators and using Kinetex PS C18 columns from two different lots. For each validation series, six calibration and six validation samples were prepared and analyzed in duplicate and quadruplicate, respectively. Calibration curves were prepared by spiking depleted serum samples at 6 levels of concentration in specific ranges for each analyte based on literature review or, in case of never reported compounds, on preliminary experiments performed during method development (concentration details in Supplementary Material Table S1). Such calibration samples were also spiked with 25 µL of IS-Mix and extracted following the protocol described in Section 2.2. Linear calibration curves were calculated from the peak area ratio of the quantifier analyte's transition to that of the corresponding IS using a $1/x^2$ weighted regression. The criteria adopted for accepting the calibration curves were a determination coefficient (R^2) > 0.98, and a ±15% maximum deviation from nominal concentration for all calibrator levels except for LLOQ where the maximum accepted deviation was set to ± 20%. In addition to the other quantitative performance indicators, combined measurement uncertainty (uc) was also calculated. The latter was obtained in accordance with the WADA Technical Document TD2021DL by quadratic combinations of the intermediate precision and the root mean square of the bias estimates [29]. For this parameter, an acceptance criterion was set at 20% of the mean result at each concentration level of validation samples. Robustness was assessed by evaluating the impact of minor changes (e.g., operator performing sample extraction, mobile phase preparation, instrument maintenance, LC column lot) introduced during the three days of quantitative validation protocol. Carry-over was assessed by injecting three extracted negative controls immediately after the most concentrated calibration sample, considering negligible carry-over when analytes' peak area in depleted serum samples were lower than 1% of the one measured in the calibration sample. Finally, the stability of extracts was assessed by preparing and analyzing three samples of a female (QCF) and male (QCM) serum pool on the same day of the extraction and after 3 and 6 days of storage at 4 °C. The same approach was also used for evaluating the stability of extracted calibration curves, using the latter extracted during the first series of validation and then stored for 3 and 6 days at 4 °C for quantifying newly extracted QCF and QCM samples.

2.5. Real serum samples application

Real serum samples were collected from healthy volunteers, 40 males (mean age 47.7 ± 14.1 years) and 40 females (mean age 38.9 ± 14.9 years), who were recruited at the Blood Bank of the City of Health and Science University Hospital of Turin. To be enrolled in the study, the volunteers had to meet the following inclusion criteria: 1) blood donor between the ages of 18 and 65 years; 2) body weight no less than 50 kg; 3) systolic blood pressure must not exceed 180 mmHg and the diastolic blood pressure should not exceed 100 mmHg; 4) hemoglobin should not be less than 12.5 g/dL in females and 13.5 g/dL in males; 5) to be healthy (not suffering from diabetes, cardiovascular diseases, epilepsy, neoplasms, autoimmune diseases). The study protocol was approved by the local ethics committee (Protocol n. 488,789).
Serum samples were collected between 7:30 and 9:00 a.m. in fasting state using SST™ II advance BD Vacutainer serum separator tubes (Becton, Dickinson & Company, Franklin Lakes, USA) in the 6 mL format. Samples were immediately sent to the laboratory after the collection and centrifuged at 1500 g for 10 min. Following 15 min of stabilization at room temperature, serum aliquot was transferred in screw cap 5 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) that were stored at −80 °C until the day of analysis.

3. RESULTS & DISCUSSION

3.1. Method development

The UHPLC-MS/MS method presented in this work aims at measuring the circulating levels of endogenous steroid hormones as well as glucuro- and sulpho-conjugated phase II metabolites, obtaining a broad overview of steroid compartment that could be useful in the detection

of EAAS doping. The selected steroid panel is composed of free glucocorticoids, progestogens, androgens mainly produced by the adrenal cortex and the gonads, that represent the basis of clinical investigations on steroid compartment. Furthermore, a central role was reserved to testosterone, considering its intermediate compounds on the way for the synthesis of androsterone and etiocholanolone, and including also glucuro- and sulpho-conjugated metabolites originated through hepatic metabolization that have already proven their usefulness as EAAS doping blood markers [19]. The rationale behind the selection of such target analytes is represented by the need of developing an analytical tool to be used in anti-doping context not only immediately in routine doping control analyses of the BSP, currently including only T, DHT and androstenedione, but also in the future for the investigation of different potential confounding factors that could have a significant impact on reported EAAS doping markers. The developed methodology aims at guaranteeing a complete overview of steroidogenesis, expanding the measured compounds in comparison with recently published works, which alternatively focused their efforts on the measurements of free steroids [18,20,22], androgens and their metabolites [24], and improving the separation and detection capabilities of previously developed extended steroid panel [23]. The inclusion of clinical endocrinology markers, such as 21-DF, DOC and 17α-hydroxyprogesterone, in target analytes list is motivated by the need of furnishing to additional information for a better understanding of both exogenous and endogenous perturbations of steroid metabolism as well as for a more accurate differentiation of abnormal steroidal markers concentrations due to prohibited substances' abuse from the ones due to pathological conditions.

The main challenges faced during method development were represented by the separation of the high number of isomeric and isobaric compounds and by the optimization of a sample preparation procedure. The latter should be capable of achieving satisfactory extraction recoveries and matrix effects for compounds that share the cyclopentaphenanthrene skeleton but own a great variety of substituents leading to significant differences in their physicochemical properties, particularly in terms of polarity. The first investigated aspect was the chromatographic separation, with the aim of obtaining baseline-separated peaks for all the 30 steroidal compounds initially included in the method. Initial gradient optimization was carried out employing the chromatographic conditions that brought recent significant improvements during previous studies on extended steroid profiling in serum [30]. LC system was equipped with Kinetex C18 column (150 × 2.1 mm, 1.7 µm, Phenomenex) and a multistep gradient was optimized using H_2O and MeOH both containing 5 mM ammonium formate as mobile phases. The addition of mobile phase modifier was indeed evaluated as an important parameter when using C18 LC columns because, thanks to the role of ammonium ions in mobile phase additive, it was possible to obtain symmetric peak shape for all sulpho-conjugated compounds. Although the separation of the different groups of isomers belonging to the class of endogenous steroids was satisfactory, with these initial conditions it was not possible to efficiently separate the two pairs of 5-Adiol glucuronides (5ααβ-Adiol 3-G/5βαβ-Adiol 3-G and 5ααβ-Adiol 17-G/5βαβ-Adiol 17-G) and the pair of sulpho-conjugated metabolites with EpiA-S and DHT-S (data not shown). Furthermore, a sensitivity issue related to all conjugated steroids detected in negative ionization mode was encountered when using mobile phases with ammonium formate buffer. For this reason, it was decided to improve negative ionization testing a different ammonium-based buffer as mobile phase modifier to increase the pH of the mobile phases. Different concentrations of ammonium acetate buffer were added to H_2O and MeOH and finally a 10 mM concentration was employed for further method development, guaranteeing sufficient sensitivity for analytes detected in negative ionization mode and satisfactory peak shape of sulphated steroids. As shown in Fig. 2A, the optimized conditions were able to separate the 5ααβ-Adiol 17-G/5βαβ-Adiol 17-G pair but did not reach the goal of separating the other two challenging pair of isomers, which were eluted together, and therefore Kinetex C18 column was discarded. It was therefore decided to test a different C18 column, the Zorbax Eclipse Plus C18 column (150 × 2.1 mm, 1.8 µm, Agilent Technologies), which already proved to be able to efficiently separate sulphated steroids in urine matrix [31]. The gradient was slightly modified in

order to optimize as much as possible the resolution of isomers' peaks but, as shown in Fig. 2B, even with this column it was possible to achieve a satisfactory separation only for 5αaβ-Adiol 17-G/5βαβ-Adiol 17-G and Etio-S/A-S pairs of isomers. An improvement in the resolution of EpiA-S and DHT-S was observed employing Zorbax Eclipse Plus C18 column, however the overall outcome of the test was considered similar to the one obtained with the Kinetex C18 column and also this second tested column was discarded. Since these core-shell and fully porous C18 columns, solely basing the chromatographic separation on hydrophobic interactions, were not able to separate conjugated steroids isomers, the use of a C18 column owning also additional retention mechanism was investigated. In this context, the Kinetex PS C18 (150 × 2.1 mm, 2.6 µm, Phenomenex), which owns a 100% aqueous stable C18 phase with a positive surface charge that allows optimal retention of polar compounds, was evaluated. Owing to the different chemistry of its stationary phase, this latter column dramatically improved the separation of 5αaβ-Adiol 3-G/5βαβ-Adiol 3-G as well as EpiA-S/DHT-S pairs of isomers (Fig. 2C), finally allowing the baseline separation of all the target analytes included in the method. The optimized chromatographic conditions are described above in Section 2.3 and the chromatograms obtained by injecting an extracted Level 3 calibration sample containing all target is presented in Fig. 3.

Once chromatographic conditions were set, method development focus was shifted to the sample preparation protocol with the purpose of obtaining a high-throughput procedure, employing 96-well SPE plates, that could be also partially automated. The extraction conditions optimized in Ref. [30] were used as starting point for sample clean-up procedure optimization. Initially, two different SPE sorbents capable of extracting neutral, acidic as well as basic compounds, were tested using the already available protocol. Performance in terms of extraction recovery and matrix effect of Strata-X (Phenomenex) and EVOLUTE® EXPRESS ABN (Biotage) 30 mg 96-well plates were compared (data not shown). The results of these preliminary experiments showed that both SPE plates were able to extract target steroidal compounds from serum matrix, with extraction recoveries ranging from 78 to 98%, but extracts obtained with Strata-X plates showed higher values of matrix effect for sulpho-conjugated analytes ranging from 112 to 146%. It was therefore decided to continue extraction optimization employing EVOLUTE® EXPRESS ABN plates. Since the protocol did not efficiently extract sulphated steroids, in the present study efforts were made to increase recoveries of such analytes maintaining negligible matrix effect for all target compounds. With this aim in view, the use of different solvents during sample dilution, washing and elution steps were tested. The use of pure deionized water for sample dilution was compared with the use of aqueous solutions containing increasing percentages of H_3PO_4 that should guarantee the disruption of binding between steroid analytes and serum proteins. Regarding the washing step of the SPE protocol, four different solvents were used starting from pure deionized water. The addition of 10% of MeOH and/or 0.1% NH_4OH was investigated as the MeOH could help in cleaning the sample removing more polar interfering compounds, while the addition of ammonium ions could, as explained for chromatographic retention, aid in the formation of ion-pair with sulphated steroids therefore increasing their interaction with the sorbent. Finally, pure MeOH and ACN were tested as elution solvents together with mixtures of the two organic solvents in 1/1 and 9/1 proportion, respectively. The results of such experiments obtained for T, T-G and T-S are presented in Fig. 4 as a useful paradigm of the behavior of the three different classes of analytes included in the method. Observing the signal intensity obtained with the different dilution solvents, it was clear that the addition of H_3PO_4 significantly increased signal intensity of all classes of steroids in the extracts, obtaining the maximum result at 2% concentration. Less significant variations were instead reported changing washing solvents, with all tested conditions leading to satisfactory results. For this step it was decided to use H_2O because, in addition to be the solvent leading to best results it was also the less complex to prepare and handle. Finally, regarding the elution step, different situations were observed for endogenous free steroids and conjugated metabolites. Indeed, the results obtained for free steroids showed similar results in all tested conditions, while the mixture of the two organic

solvents allowed higher recovery rates for more polar compounds such as glucuro- and sulpho-conjugated steroids. The final optimized sample extraction protocol is described in Section 2.2 and the extraction recoveries and matrix effects obtained for all target analytes are summarized in Table 1.

3.2. Method validation

An extensive quantitative validation protocol was set up in accordance with the ISO/IEC and WADA requirements. Before starting the validation experiments, preliminary analysis of a small group of real serum samples were carried out with the aim of evaluating the presence or absence of all 30 steroidal compounds initially included in the developed method. During such preliminary studies two analytes, E-G and 5αββ-Adiol 3-G, were never detected in both male and female samples and were therefore excluded from method validation. Furthermore, a significant interference of DHEA-S on 5αββ-Adiol 17-S transition was observed in all analyzed serum samples due to fact that the precursor ion monitored for 5αββ-Adiol 17-S (*m/z* 371.0), coincides with an isotopic peak of negatively charged DHEA-S, which accounts for approximately the 0.1% of the total abundance of that analyte. Since DHEA-S is circulating at higher concentrations than 5αββ-Adiol 17-S (low µg/mL vs low ng/mL ranges) and that the two compounds, which are not resolved at baseline, own the same product ion characteristic of all sulphated compounds in negative ionization mode ([HSO4]-, *m/z* 97.0), the signal of the less abundant isotope of DHEA-S causes the observed interference. Because of the impossibility of solving the above-mentioned issue, although detectable peaks of 5αββ-Adiol 17-S were observed in most of analyzed serum samples, it was preferred to take out this analyte from quantitative validation.

Regarding the selectivity, the developed SPE-based sample preparation protocol together with the optimized chromatographic separation and the selection of up to two MS/MS transitions for each target analyte provided a first level of selectivity to the method. Details on selected MRM transitions and retention times can be found in Supplementary Material Table S3 and Table 1, respectively. The absence of chromatographic interferences in the selected MRM transitions of all target analytes was verified by analyzing five negative control serum samples and five negative control serum samples spiked with a solution containing approximately 60 exogenous steroids at a concentration between 2 and 5 ng/mL. The observation of MRM chromatograms of negative sera in the elution region of each steroid did not show any notable interferences (<20% LLOQ). Furthermore, ten real samples (5 males and 5 females) were also analyzed in "Product Ion Scan" mode with a fixed collision energy, comparing the obtained MS/MS spectra with a Level 4 calibration sample. Real serum samples as well as calibration samples were also analyzed with the developed method comparing the measured fragment ion ratio, calculated by dividing the area of the peak obtained in the two MRM transitions, and for all target steroids the results obtained in both female and male samples was comprised in the acceptance range (data presented in Supplementary Material Table S4). The comparison of IS peaks' areas in serum samples showing different levels of hemolysis did not highlight significant signal enhancement/suppression, with measured areas not exceeding 2 standard deviations for all available IS (the plots with the results obtained for all IS are presented in Supplementary Material Figs. S2–S4). The investigation of chromatograms obtained from serum pool samples with different hemolysis levels and negative serum samples spiked with Level 5 calibration solution resulted in no significant interference observed in the region of elution of all target analytes in selected MRM transitions (chromatograms obtained in pool sample with [Hgb] between 200 and 299 mg/dL are presented in Supplementary Material Figs. S5–S7). The analysis of IS-mix diluted 1:5 in MeOH/H2O 1/1 (v/v) solution, allowed to assess the absence of any notable interference (<20% LLOQ) in the MRM transition of all target analytes (obtained chromatograms presented in Supplementary Material Figs. S8–S9). Thanks to the optimized sample clean-up procedure, satisfactory extraction recoveries ranging from 84.7% (EpiA-S) to 97.9% (5βαβ-Adiol 3-G) and matrix effects ranging from 89.6 (EpiA-S) to 107.2% (Etio-S) were obtained using the methodology proposed by Matuszevsky et al. [28]. A summary of all quantitative validation results is presented in Table 1, together with target

analytes retention times and measured extraction recoveries and matrix effects. The quantitative validation protocol, carried out in three analytical series by two different operators analyzing the six level of calibration curve in duplicate as well as the six levels of validation samples in quadruplicate, assessed satisfactory trueness and precision values for all target analytes.

More in detail, precision, displayed in Table 1 as repeatability, and intermediate precision values ranged from 1.5 to 8.6% and from 2.8 to 8.3%, respectively. As expected, the precision was inversely proportional to analytes' concentration. As requested by WADA technical document TD2021DL, combined uncertainty, which considers both random (precision) and systematic errors (accuracy) by combining random and systematic error, was also calculated at six concentration levels, showing values that ranged from 4.6 to 12.8% and being for all analytes at all concentration levels below the predefined threshold acceptance value of 20%. The LLOQ of each analyte was defined as the lowest concentration for which a combined uncertainty lower than 20% was measured and coincided for all target steroidal compounds with the lowest concentration of the calibrators/validators (uncertainty profiles presented in Supplementary Material Figs. S10–S12). The obtained LLOQ ranged from 20 pg/mL (E) to 200 ng/mL (DHEA-S), highlighting the variety in circulating levels of measured steroid hormones and the necessity of high-level MS instrumentation owning a wide dynamic range. Furthermore, analyzing more in detail the LLOQ values calculated for each target steroid, it possible to note that the developed method showed a satisfactory sensitivity level for most of selected analytes. LLOQ obtained for P, DHT and DHT-S, could be considered as satisfactory for measuring these analytes in female (P) and male healthy population [32,33], but specific ultra-sensitive assays should be employed to measure such hormones in other populations. A separate discussion should be instead made for DOC and 21-DF, for which the obtained LLOQ could not guarantee to measure their serum concentration in the majority of healthy individuals, but it is sufficient to highlight increased values that could occur in presence of endocrine disorders such as Congenital Adrenal Hyperplasia and adrenal tumors [34,35]. Negligible carry-over was observed for all target analytes, with values below 0.1% for most of target analytes except for DHEA-S (0.8%), for which the most concentrated calibration sample was at 12 μg/mL. Method's robustness was assessed during the three days of quantitative validation protocol, employing two different operators across performed batch of analyses; different mobile phases and solutions for SPE extraction were prepared for each day, analytical LC columns of two different lots were used and, in addition, instrument maintenance (ESI source cleaning) was performed before each analytical batch. With all these variations, the calibration lines (presented in Supplementary Material Table S5) were satisfactory with an $R^2 > 0.98$ and the measurement of uncertainty performed gave values lower than 20% for all target analytes compounds. Therefore, the developed method was considered robust in the range of linearity for each compound.

The stability of the extracted samples was assessed by storing the three QCF and QCM aliquots of the first validation series (day 0) at 4 °C and reinjecting them three days later (day 3). Then, the aliquots of the second validation series were stored at 4 °C and reinjected six days later (day 6). The concentrations of the QCF and QCM samples extracted during day 1 were compared to the ones stored for three and six days at 4 °C. The quantification results, presented in Supplementary Material Tables S6–S8 and showing concentration differences lower than 15% for all detected analytes. This finding proved that the extracted compounds are stable in collection plates for at least five days at 4 °C, which is a very useful information for the application of the developed method in routine anti-doping laboratory work (e.g., instrument maintenance or problems). Furthermore, to evaluate the possibility of reusing an extracted calibration curve for more than one analytical batch of analysis, the three aliquots of QCF and QCM samples extracted during the first validation series were quantified using the calibration curve samples extracted on the same day and the calculated values were used as a reference (day 0). Three aliquots of QCF and QCM samples from the second validation series were processed using data from the calibration curve of day 1, that were extracted and analyzed 3 days previously (day 3). Then, three aliquots of QCF and QCM samples from the third validation series were processed using data from the day 1 calibration curve, extracted and analyzed

6 days previously (day 6). The concentrations of QCF and QCM extracted on day 3 and day 6 were compared to the ones of day 1 and did not show concentration differences higher than 15% for all detected target analytes (details in Supplementary Material Tables S9–S11).

3.3. Real serum samples application

Serum samples collected from 80 healthy volunteers were randomized and analyzed in four different analytical series during a one-month period. To evaluate the accuracy of the measured concentrations, in each analytical series three external quality controls (QC, MassChrom® Steroids in Serum/Plasma, Chromsystems, Gräfelfing, Germany), containing twelve endogenous steroids and DHEA-S at three different concentrations included in the linearity range of the method, were also prepared and analyzed together with volunteers' samples. For all four analytical sessions, the concentrations of the analytes present in QC samples did not deviate from nominal concentration for more than 15%. The goal of the application of the developed and validated method to real serum samples was to estimate normal reference ranges of the serum concentrations of the 27 target steroid hormones and androgens' phase II metabolites. For this purpose, normality of the distributions of steroids concentrations measured in male and female populations was assessed using the Shapiro-Wilk test. In Table 2 reference intervals obtained for all target steroidal compounds in both investigated populations are reported with the mean concentration and the standard deviation in case of normal distributions and with the median concentration and the interquartile range (IQR, 25th and 75th percentile) for not normal distributions.

Observing the obtained results, it is possible to notice that three endogenous steroids (21-DF, DOC and E) were not detected in most of the analyzed female and male samples, therefore resulting in the absence of useful reference intervals. Such outcome highlighted the need of more sensitive analytical approaches for the detection of these compounds in serum and at the same time their difficult introduction in a longitudinal monitoring. Similar conclusions could be drawn for P, which concentrations were below method LLOQ (50 pg/mL) in most of male samples, and for some of androgens' phase II metabolites such as T-G, DHT-S, E-S and T-S, which were less abundant in female samples with concentrations below method's LLOQ (50, 100, 50 and 100 pg/mL, respectively). Regarding all other endogenous steroid hormones, the results obtained in this application were in accordance with the most recent literature dealing on the evaluation of reference range of the target hormones measured by LC-MS in clinical context [[36], [37], [38]]. Discussing the results obtained for phase II metabolites, it is possible to appreciate the additional information that the developed method could furnish in the field of steroid analysis. Indeed, we were able to describe for the first time to our knowledge the distribution of different circulating isomers of 5-Adiol glucuronide. More in detail, it was observed that all four detected isomers were more concentrated in male population, with the 17-glucuronidated forms showing higher circulating concentrations in men. Such result was not observed in the female population where 5ααβ-Adiol 17-G was found to be less abundant than 5ααβ-Adiol 3-G. Since these analytes were already reported as sensitive markers of oral T doping, the described findings could represent a fundamental reference for normality in the context of doping control analyses, and furthermore could also be relevant for endocrinological purposes. In fact, the routine measurement of the sum of 5-Adiol glucuronide isomers as a marker of androgen activity [39] could potentially take advantage of the efficient separation of all circulating isomers that may be used to investigate pathological conditions for which there is still lack of measurable indicators, such as idiopathic hirsutism. Further novel information gathered by the presented application is represented by the efficient separation of EpiA-S and DHT-S. The latter was measured only in the male population with a median concentration of 1 ng/mL (0.34–1.59 ng/mL), representing a novelty in the field since its serum concentrations were never reported to date. This result is in accordance with previous study by Sanchez-Guijo et al. [40] that investigated sulphated steroid metabolism by LC-MS analysis with a LLOQ for DHT-S at 1 ng/mL and did not detect DHT-S in all analyzed plasma samples. Among the other sulpho-conjugated androgens metabolites, it is worth to mention that for more concentrated analytes such as A-S, DHEA-S and EpiA-S, reference intervals

in agreement with the previous literature were found [40,41], measured serum concentrations of these analytes, ranging from hundreds of ng/mL to low µg/mL, confirm the possibility of their future use as sensitive markers (A-S and EpiA-S) as wells as endogenous reference compounds (DHEA-S) for the implementation of a IRMS-based confirmatory analysis in the framework of BSP [26].
This study owns however a limitation represented by the low number of samples collected in both male and female population and the obtained results will therefore need further validation with larger study cohorts. Nevertheless, with the presented research work it was possible to test the reliability and robustness of the developed and validated analytical method as well as to give an innovative insight on androgens metabolism.

4. CONCLUSIONS

Blood steroid profiling is currently consolidating its position among other approaches in the analysis of anti-doping samples, representing an alternative for the detection of EAAS doping and a complementary platform to the urinary steroidal module of the ABP. The aim of this research work was to strengthen the approach to accurate quantitative analysis of an extensive set of markers of EAAS doping in blood. We developed a single-run UHPLC-MS/MS method for the simultaneous measurement in serum and plasma matrix of 27 steroidal compounds (13 endogenous steroid hormones, 7 glucuro-conjugated and 7 sulpho-conjugated phase II androgen metabolites). The main advantage of the presented method, in comparison with previously reported assays in the anti-doping field, lies in the ability of separating challenging isomeric and isobaric compounds, such as four isomers of 5-Adiol glucuronide and the pair of EpiA-S/DHT-S. Chromatographic resolution of such glucuronide isomers, in contrast with the monitoring of the total amount of 5ααβ-Adiol and 5βαβ-Adiol glucuronides performed in routine urinary analyses, could be of outmost importance to evaluate the different pathways involved in the metabolization of exogenous administered T. Indeed, as already showed for UGT2B17 enzyme, the effects of enzymatic polymorphism could lead to significant differences in metabolic pathways of androgens' glucuronides that may affect their performance as EAAS doping markers [6,7]. Furthermore, the obtained unambiguously differentiation of EpiA-S and DHT-S, detected in a single chromatographic run and alternatively reported in previous researches for anti-doping purposes [19,24], represents an interesting novelty and a precious anchor point for further studies on BSP. The fine chromatographic optimization needed to obtain baseline separation of all target analytes resulted in a total run time of 25 min. The creation of such a long LC gradient surely represents a drawback for the routine implementation of the presented methodology. However, the obtained separation makes the developed assay a strategic tool to be employed in further investigations on BSP that could finally lead to the selection of a limited number of EAAS doping blood markers to be monitored with dedicated faster and more sensitive methods. Sample preparation procedure was also optimized by comparing different solvents for sample dilution as well as for SPE washing and elution steps. Final protocol consists of 7 steps that, with the aim of reducing operator working time and analytical error sources, could be partially automated thanks to dedicated robots for liquid handling and positive pressure application. Although there are significant differences in physicochemical properties between the steroids of interest, the developed protocol resulted in satisfactory extraction recoveries (from 84.7 to 97.9%) for all target analytes, without considerable matrix effects (from 89.6 to 107.2%). This aspect constitutes a significant advancement if compared with the performance of other analytical solutions reported in literature for conjugated steroid analysis [23,24,30]. The method was validated in accordance with WADA regulations for quantitative analyses, demonstrating acceptable results in terms of selectivity, trueness, repeatability, precision, combined uncertainty, linearity range, LLOQ, carry-over and stability.
Furthermore, the analytical platform was employed for investigating the normality ranges of target analytes in a healthy population of 40 females and 40 males. This preliminary study proved the developed method robust and reliable, providing normality ranges in accordance with the literature

for most of the investigated compounds. In addition, it allowed for describing for the first time the distribution of 5-Adiol glucuronide isomers in blood for the first time, with the 17-glucuronidated forms being the most abundant in both female and male populations. A further major outcome of such study was the quantification of DHT-S in male serum samples. This finding confirmed the hypothesis that such steroid, for which serum concentrations were never reported to our knowledge, is circulating in blood at low concentrations, therefore stressing the importance of its unambiguous separation from its highly abundant isomer, EpiA-S.
In the near future, we trust that the developed method could be useful for studies exploring the potential of blood steroid profiling for the detection of EAAS doping. In addition to this, it could represent a precious analytical tool for the investigations on confounding factors such as plasmatic volume, physical exercise, alcohol consumption, circadian rhythm and intake of therapeutic drugs (e.g., birth control pills, corticosteroids, 5α-reductase inhibitors, miconazole and others) that were not investigated in previous studies and could have a huge impact on the evaluation of BSP as it is already the case with urinary steroidal module of ABP. Moreover, considering the strength of the extended steroid profiling, the application to the clinical context could offer a significant step forward for research focused on understanding the steroidal disorders due to endocrinological and metabolic diseases.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Summary of quantitative validation results.

Compound	rT [min]	Trueness [%]	Repeatability [%]	Intermediate Precision [%]	Combined Uncertainty [%]	Linearity Range [ng/mL]	LLOQ [pg/mL]	Extraction Recovery (CV) [%]	Matrix Effect (CV) [%]
11-Deoxycortisol	7.98	97.1–104.3	3.9–7.3	4.7–6.9	7.3–10.8	0.05–15	50	88.4 (1.1)	101.9 (1.6)
17α-Hydroxyprogesterone	10.99	95.3–104.4	4.7–6.1	5.4–6.2	8.0–9.2	0.1–15	100	91.6 (2.5)	102.8 (4.1)
21-Deoxycortisol	7.05	98.1–105.4	5.0–8.3	5.3–7.6	7.3–12.0	0.1–10	100	91.3 (1.9)	100.0 (2.7)
Androstenedione	9.33	96.2–103.5	4.1–8.5	4.7–8.2	7.5–11.4	0.1–15	100	85.5 (1.7)	103.2 (5.8)
Corticosterone	7.65	98.4–105.0	5.5–7.3	5.1–7.8	8.7–11.2	0.2–50	200	89.9 (1.6)	97.3 (2.9)
Cortisol	5.79	98.8–103.8	4.2–6.8	5.7–6.6	8.2–9.5	10–500	10'000	91.0 (0.9)	101.4 (4.8)
Cortisone	5.25	96.5–103.0	2.0–7.4	5.0–7.6	7.4–10.7	1–50	1'000	92.5 (1.8)	98.6 (2.1)
Deoxycorticosterone	10.09	95.2–106.1	4.0–7.8	4.8–7.1	8.3–10.4	0.1–6	100	90.9 (2.3)	102.5 (2.9)
DHEA	10.65	97.3–104.1	3.7–8.6	4.8–7.9	7.0–11.6	0.5–60	500	93.7 (3.7)	102.7 (6.8)
DHT	12.71	98.8–103.3	3.7–5.8	4.5–6.7	6.4–9.4	0.05–5	50	93.6 (7.5)	103.0 (3.2)
Epitestosterone	12.29	96.0–104.1	4.5–7.0	5.4–7.4	8.0–10.4	0.02–10	20	92.9 (1.9)	100.3 (4.3)
Progesterone	13.91	94.8–103.4	3.2–6.7	4.2–6.1	7.5–9.1	0.05–25	50	91.0 (2.1)	102.0 (3.4)
Testosterone	10.25	98.5–104.9	3.1–7.4	4.6–7.2	4.6–6.9	0.05–15	50	91.4 (1.9)	102.7 (2.5)
5αaβ-Adiol 17-Glucuronide	8.32	97.0–102.7	3.3–6.7	5.2–6.7	7.2–9.4	0.05–20	50	95.2 (2.7)	101.7 (5.6)
5αaβ-Adiol 3-Glucuronide	7.47	98.3–106.5	2.7–7.3	3.3–7.2	6.1–10	0.05–10	50	96.4 (4.9)	98.3 (2.7)
5βaβ-Adiol 17-Glucuronide	8.59	98.4–104.2	3.4–7.5	5.5–8.3	7.7–12.2	0.05–20	50	96.3 (3.6)	95.6 (4.2)
5βaβ-Adiol 3-Glucuronide	7.25	96.7–101.8	3.8–7.5	4.8–8.1	6.9–11.4	0.05–10	50	97.9 (0.7)	103.5 (5.7)
Androsterone Glucuronide	8.52	100.6–103.1	1.9–6.3	3.5–5.8	5.6–8.7	0.5–75	500	96.3 (5.3)	93.7 (7.6)
Etiocholanolone Glucuronide	7.99	99.8–103.9	3.6–6.2	5.0–7.0	7.0–9.7	0.5–75	500	96.8 (6.7)	99.5 (3.1)
Testosterone Glucuronide	5.02	97.5–103.6	3.5–6.9	4.2–6.3	6.2–9.1	0.05–10	50	91.6 (2.3)	99.6 (4.4)
Androsterone Sulphate	8.76	93.9–104.6	3.2–4.7	3.4–5.4	7.0–8.5	5–2500	5'000	90.9 (4.8)	105.8 (7.7)
DHEA Sulphate	6.35	95.5–106.8	3.1–5.8	3.6–6.2	6.0–9.5	200–12000	200'000	90.8 (8.1)	98.3 (9.8)
DHT Sulphate	6.97	91.7–106.3	2.8–6.1	3.4–6.8	6.9–11.8	0.1–10	100	87.7 (3.2)	102.6 (5.2)
Epiandrosterone Sulphate	6.72	97.2–105.0	2.3–6.3	2.8–6.4	5.8–9.2	5–2500	5'000	84.7 (2.2)	104.9 (4.3)
Epitestosterone Sulphate	7.01	94.4–104.2	2.7–5.1	3.0–6.2	6.7–9.5	0.05–10	50	88.3 (1.8)	89.6 (6.2)
Etiocholanolone Sulphate	8.39	94.0–102.9	2.1–6.3	3.5–6.2	5.6–9.2	5–2500	5'000	88.9 (8.6)	107.2 (9.0)
Testosterone Sulphate	6.03	94.2–106.1	1.5–5.6	2.9–6.4	6.9–10	0.1–20	100	88.4 (3.0)	90.9 (7.4)

Table 2. Reference intervals of all target analytes in healthy male and female populations.

Compound	Serum concentrations [ng/mL] Median (IQR, Q1-Q3)/Mean (SD)	
	Males (n = 40)	Females (n = 40)
11-Deoxycortisol	0.18 (0.12–0.35)	0.18 (0.09–0.29)
17α-Hydroxyprogesterone	0.62 (0.28)	0.18 (0.10–0.70)
21-Deoxycortisol	<0.1	<0.1
Androstenedione	0.50 (0.21)	0.67 (0.40)
Corticosterone	2.48 (1.78–3.37)	2.66 (1.89–6.38)
Cortisol	121.1 (104.4–141.0)	144.4 (104.2–177.9)
Cortisone	17.1 (15.4–19.2)	19.4 (3.8)
Deoxycorticosterone	<0.1	<0.1
DHEA	1.62 (1.16–2.42)	2.23 (1.67–2.88)
DHT	0.37 (0.17)	<0.05
Epitestosterone	<0.02	<0.02
Progesterone	<0.05	0.06 (0.05–0.19)
Testosterone	5.13 (1.86)	0.23 (0.11)
5αaβ-Adiol 17-Glucuronide	1.36 (0.85–2.12)	0.29 (0.14–0.51)
5αaβ-Adiol 3-Glucuronide	1.21 (0.83–1.54)	0.68 (0.28–1.07)
5βaβ-Adiol 17-Glucuronide	3.75 (2.43–5.00)	1.25 (0.65–2.85)
5βaβ-Adiol 3-Glucuronide	0.54 (0.36–0.90)	0.29 (0.18–0.43)
Androsterone Glucuronide	29.0 (22.5–37.7)	17.7 (9.4–23.8)
Etiocholanolone Glucuronide	19.2 (13.6–27.1)	13.1 (9.2–22.0)
Testosterone Glucuronide	0.27 (0.19–0.35)	<0.05
Androsterone Sulphate	376.4 (21.2)	285.9 (174.4–478.1)
DHEA Sulphate	1.45 (0.94–2.14)[a]	1.30 (0.77–1.66)[a]
DHT Sulphate	1.00 (0.34–1.59)	<0.1
Epiandrosterone Sulphate	126.7 (82.8–167.1)	68.9 (40.4–123.1)
Epitestosterone Sulphate	0.20 (0.12–0.27)	<0.05
Etiocholanolone Sulphate	15.9 (5.80–51.5)	31.8 (16.4–43.7)
Testosterone Sulphate	0.42 (0.28–0.67)	<0.1

[a]Concentrations in µg/mL.

Fig. 1. Sample preparation schematic workflow. Pre-treated blank, calibration and serum samples loaded on the SPE plate in the “Loading” step.

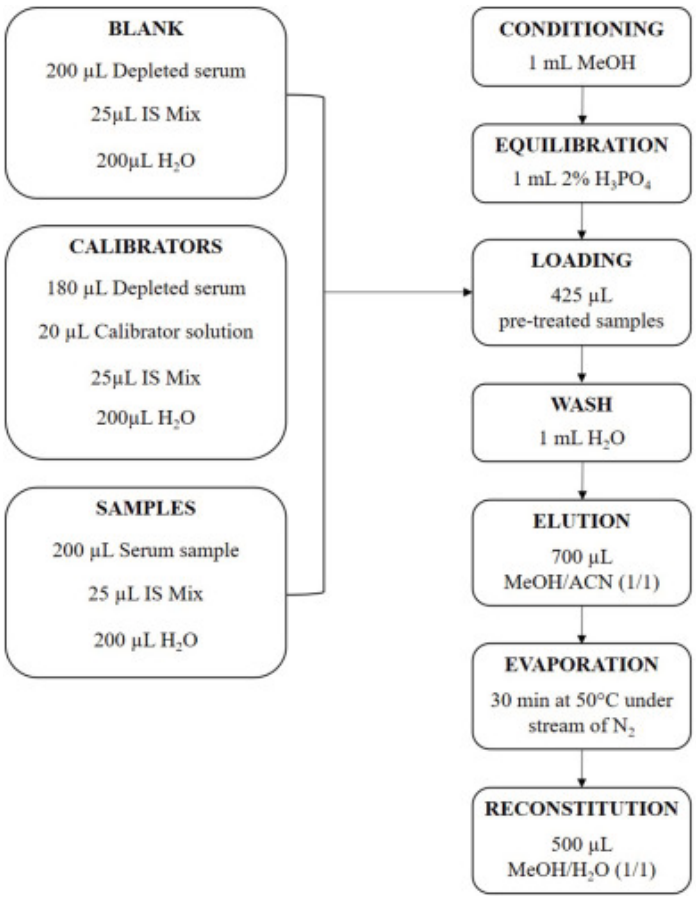


Fig. 2. Chromatograms of glucuronide isomers with *m/z* 467.1 to 112.9 and sulphate isomers with *m/z* 368.7 to 96.9 obtained using the three tested C18 columns. A) Kinetex C18 (150 × 2.1 mm, 1.7 µm; Phenomenex); B) Zorbax C18 Eclipse Plus (150 × 2.1 mm, 1.7 µm; Agilent Technologies); C) Kinetex PS C18 (150 × 2.1 mm, 2.6 µm; Phenomenex). G3: 5αββ-Adiol-3G; G4: 5βαβ-Adiol 3-G; G5: 5ααβ-Adiol 3-G; G6: 5ααβ-Adiol 17-G; G7: 5βαβ-Adiol 17-G; S5: EpiA-S; S6: DHT-S; S7: Etio-S; S8: A-S.

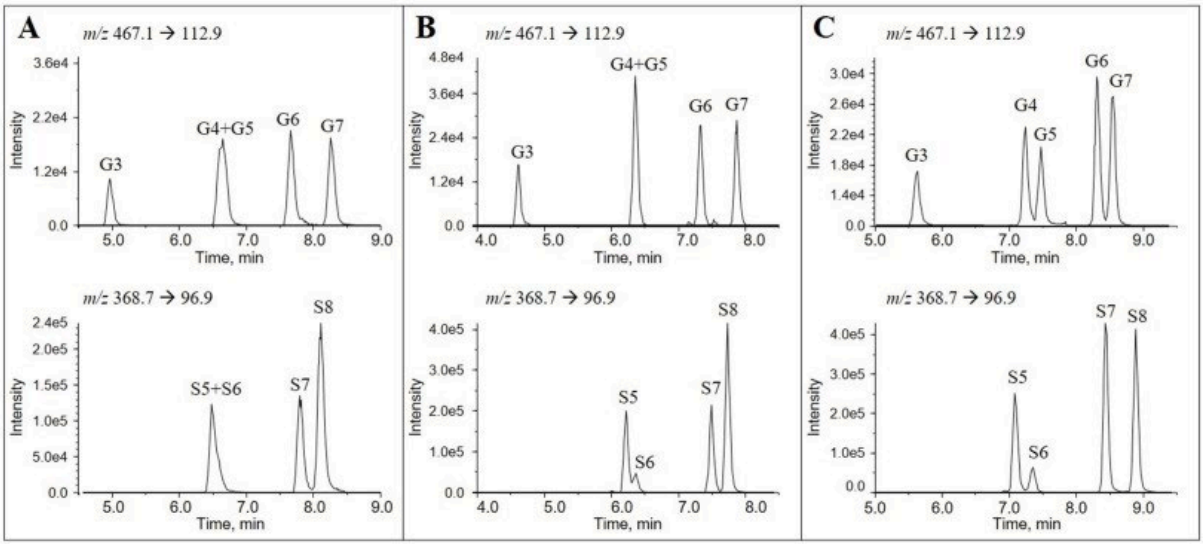
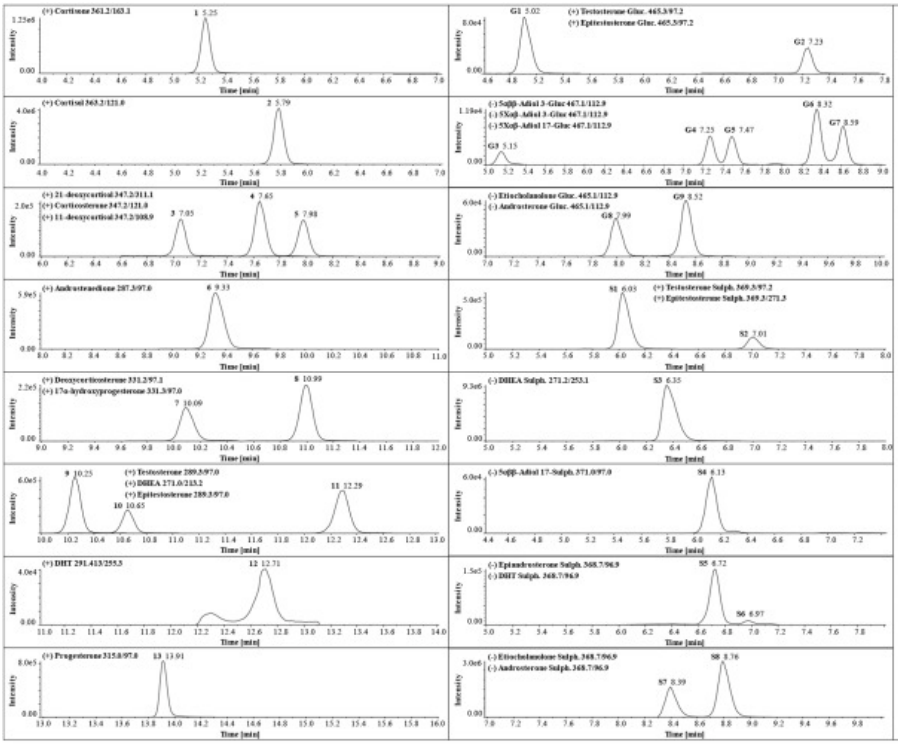


Fig. 3. Chromatograms obtained with the optimized chromatographic gradient for Level 3 calibration sample containing all target analytes.



1	Cortisone
2	Cortisol
3	21-Deoxycortisol
4	Corticosterone
5	11-Deoxycortisol
6	Androstenedione
7	Deoxycorticosterone
8	17α-Hydroxyprogesterone
9	Testosterone
10	DHEA
11	Epitestosterone
12	DHT
13	Progesterone
G1	Testosterone Glucuronide
G2	Epitestosterone Glucuronide
G3	5αββ-Adiol 3-Glucuronide
G4	5βαβ-Adiol 3-Glucuronide
G5	5ααβ-Adiol 3-Glucuronide
G6	5ααβ-Adiol 17-Glucuronide
G7	5βαβ-Adiol 17-Glucuronide
G8	Etiocholanolone Glucuronide
G9	Androsterone Glucuronide
S1	Testosterone Sulphate
S2	Epitestosterone Sulphate
S3	DHEA Sulphate
S4	5αββ-Adiol 17-Sulphate
S5	Epiandrosterone Sulphate
S6	DHT Sulf
S7	Etiocholanolone Sulphate
S8	Androsterone Sulphate

Fig. 4. Normalized signal intensity obtained employing different solvents at sample dilution, washing and elution steps for testosterone, testosterone glucuronide and testosterone sulphate.