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Steroid profiling by UHPLC-MS/MS in dried blood spots collected from healthy women with and without testosterone gel administration

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ABSTRACT

The quantification of a large panel of endogenous steroids in serum by LC–MS/MS represents a powerful clinical tool for the screening or diagnosis of diverse endocrine disorders. This approach has also demonstrated excellent sensitivity for the detection of testosterone misuse in the anti-doping field, especially in female athlete population. In both situations, the use of dried blood spots (DBS) could provide a viable alternative to invasive venous blood collection. Here, the evaluation of DBS sampling for the quantification of a panel of endogenous steroids using UHPLC-MS/MS is described.

The UHPLC-MS/MS method was validated for quantitative analysis of eleven free and eight conjugated steroids and was then used for the analysis of DBS samples collected in 14 healthy women during a normal menstrual cycle (control phase) followed by a 28-days testosterone gel treatment (treatment phase). Results were compared with those obtained from serum matrix. Satisfactory performance was obtained for all compounds in terms of selectivity, linearity, accuracy, precision, combined uncertainty, stability as well as extraction recovery and matrix effects. In control phase, high correlation was observed between DBS and serum concentrations for most compounds. In treatment phase, higher testosterone concentrations were observed in capillary than in venous DBS, suggesting a possible interference resulting from testosterone contamination on finger(s) used for gel application.

Steroid profiling in capillary DBS represents a simple and efficient strategy for monitoring endogenous steroid concentrations and their fluctuation in clinical context of steroid-related disorders, or for the detection of testosterone abuse in anti-doping.

1. Introduction

The diagnosis and the monitoring of various endocrine conditions frequently depend on the quantitative analysis of endogenous steroids in blood matrix. For many years, this analysis was performed using immunoassays allowing high-throughput. However, this technique is subject to cross-reactivity and often limited to the measurement of a single or few compounds instead of a flexible panel of substances. Actually, clinical laboratories rather use liquid chromatography-tandem mass spectrometry (LC–MS/MS) as gold standard method to measure steroid hormones, because it offers the possibility of quantifying multiple analytes at the same time with higher specificity and selectivity [1]. Along further extensions of the panel, e.g. phase II metabolites, LC–MS/MS is indeed a valuable approach for the study of potential alterations in steroidogenesis due to hormonal imbalances, especially in women for whom androgenic activity is not necessarily reflected by serum testosterone level [2,3].

In the anti-doping context, testosterone misuse is currently targeted using an individual and longitudinal monitoring of urinary biomarkers of testosterone in the so-called Athlete Biological Passport (ABP) [4]. In case of abnormal values for one or several of these markers, a time consuming and expensive analysis based on gas chromatography-combustion- isotope ratio MS (GC/C/IRMS) is performed to confirm the potential exogenous origin of testos-

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terone and its metabolites. While the implementation of this tool improved the testosterone detection capability, various confounding factors may influence the urinary steroid profile complicating its interpretation and decreasing its sensitivity [5–7]. Furthermore, athletes rather resort to low doses of topical testosterone, which significantly reduces peaks of urinary concentrations that are difficult to discriminate from natural variability [7–9].

To overcome these limitations, endogenous steroid profiling in serum has been proposed as a potential complementary approach to the urinary steroid profile for the detection of testosterone misuse by athletes [9,10]. Particularly, the blood matrix is more informative than urine for the correlation between hormone concentration and the physiological responses. The longitudinal monitoring in serum has been proven particularly useful for testosterone detection in female subjects in whom menstrual fluctuations may lead to a great source of variation for urinary biomarkers disrupting their sensitivity [7,9,11].

A primary drawback of the application of serum steroid profiling is that it requires invasive venous blood sampling and sample collection by a trained phlebotomist. Moreover, these biological specimens have to be transported under cooled temperature conditions within a short timeframe, which all increase the total costs of sample collection. Dried blood spots (DBS), which are based on the transfer of a limited volume of capillary blood onto a filter paper or similar matrix, could tackle these obstacles offering a convenient and more affordable alternative. This process benefits from minimal invasiveness, simplicity of sample collection, facilitated transport and storage conditions, and reduced costs that could allow for more frequent sampling for anti-doping programs. The advent of volumetric microsampling technologies has further improved the collection of DBS for quantitative purposes. While DBS have been used for neonatal screening for decades, its applicability has been recently evaluated for SARS–CoV–2 serology assays [12], therapeutic drug monitoring [13] or alcohol abstinence [14]. In the anti-doping context, DBS have been considered as complementary matrix for many years [15–18] and have been investigated for either direct detection of prohibited substances [19–21] or indirect detection through potential biomarkers [22–24]. In particular, a method using volumetric microsampling and GC–MS/MS was recently developed for the quantification of testosterone and eight synthetic anabolic androgenic steroids (AAS) [25]. However, this method was limited to the quantification of only one endogenous AAS (EAAS) and could therefore be hardly applied in the clinical context for the monitoring of steroidogenesis disorders such as polycystic ovary syndrome or congenital adrenal hyperplasia.

In this study, we developed a UHPLC-MS/MS method for the simultaneous determination of eleven free (testosterone, epitestosterone, androstenedione, dehydroepiandrosterone (DHEA), 5α-dihydrotestosterone (DHT), progesterone, 17α-hydroxyprogesterone, cortisol, corticosterone, deoxycorticosterone and 11-deoxycortisol) and eight conjugated (glucuro-conjugated androsterone and etiocholanolone, sulfo-conjugated testosterone, DHEA, androsterone, etiocholanolone, epiandrosterone and dehydroandrosterone) steroids in DBS matrix. Following validation according to World-Anti Doping Agency (WADA) requirements, the method was applied to the analysis of DBS samples collected from healthy eumenorrheic women during a normal menstrual cycle followed by a 28-days T gel treatment and results were compared with those of serum.

2. Materials and methods

2.1. Chemical & reagents

Methanol (MeOH) was purchased from Macron Fine Chemicals (Deventer, The Netherlands), formic acid (FA; UHPLC/MS, >99 %) and UPLC grade MeOH from Biosolve BV (Valkenswaard, The Netherlands) while ammonium hydroxide (NH_4OH; 28–30 %) solution was obtained from Sigma-Aldrich (Buchs, Switzerland). Charcoal Dextran Stripped Human Serum was supplied by Dunn Labortechnick GMbH (Asbach, Germany). Milli Q quality water was obtained from a Milli-Q® grade system (Millipore, MA, USA) and was used for the preparation of LC mobile phases and extraction/reconstitution solutions.

Testosterone (T), androstenedione were purchased from Fluka (Buchs, Switzerland), 11-deoxycortisol, deoxycorticosterone, 17α-hydroxyprogesterone, cortisol, epitestosterone from Sigma-Aldrich (Buchs, Switzerland), dihydrotestosterone, corticosterone, dehydroepiandrosterone (DHEA) from Chemie Brunschwig (Basel, Switzerland), etiocholanolone glucuronide, androsterone sulfate, deyhdroepiandrosterone sulfate (DHEAS), testosterone sulfate, epiandrosterone sulfate from Steraloids (Newport, RI, USA), androsterone glucuronide, etiocholanolone sulfate from LGC Standards (Wesel, Germany) and progesterone from Laboratoire Golaz (Lausanne, Switzerland). Internal labelled standards (IS) were provided by National Measurement Institute (Pymble, Australia).

2.2. Sample preparation

2.2.1. Calibration curves and quality control samples

Artificial steroid stripped blood was prepared from whole blood of a healthy volunteer using a modified method reported by Higashi et al. [26]. The whole blood sample was first centrifuged at 1500 × g for 15 min and the separated plasma was discarded. The red blood cells (RBCs) were washed with saline solution, centrifuged and the supernatant was discarded. This procedure was repeated three times and the washed RBCs were then combined with charcoal stripped serum to obtain a hematocrit of 50 %.

Calibration and quality control (QC) samples were then prepared in artificial blood by spiking with reference material of each analyte (Table S1) and 20 μL of the spiked blood was spotted onto Whatman 903™ protein saver cards (GE Healthcare). The cards were dried for a minimum of 1 h at room temperature (RT) and stored at 4 °C unless used for stability study.

2.2.2. DBS samples extraction

For each sample, the whole spot of 20-μL was excised from the DBS card and transferred into a clean 1.5 mL conical polypropylene microcentrifuge tube. One milliliter of methanol/water 95:5 (v/v) containing the internal standard (IS) mixture (Table S2) was added to each tube which was then briefly vortexed and subjected to sonication for 15 min. The supernatant was transferred into a 96-well collection plate equipped with glass inserts and evaporated to dryness under nitrogen stream. The extracts were finally reconstituted in 100 μL of a MeOH/H_2O (50:50, v/v) solution, and after 10 min of gentle shaking, 10 μL of each extract was injected into UHPLC-MS/MS system.

2.3. Instrumentation and analytical conditions

An Acquity UPLC I-class (Waters, Milford, USA) equipped with a Kinetex C18 column (150 × 2.1 mm, 1.7 μm; Phenomenex, Torrance, CA, USA) was used for chromatographic separation. The mobile phase A solution was H_2O + 5 mM ammonium formate and mobile phase B was MeOH + 5 mM ammonium formate. Column temperature was set at 60 °C and separation was performed with a flow rate at 300 μL/min, applying the same gradient as described in [27]. MS/MS analysis was carried out using a Xevo-TQ S triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) source operating in polarity switching (positive/negative) mode. Multiple reaction monitoring (MRM) mode was employed using the con-

ditions described in Table S3. MassLynx software version 4.2 was used for data acquisition and TargetLynx for data processing.

2.4. Method validation

2.4.1. Selectivity

Selectivity was assessed by the analysis of steroid stripped blood specimens in ten replicates for the presence of interfering signals at the expected retention times of the target analytes.

2.4.2. Calibration curve and linearity

Target analytes were spiked at various concentration levels (Table S1) and calibration curves were established with at least five concentration levels and prepared in three separate analytical series. Linear calibration models were generated for each compound (peak area ratio of each steroid to its respective IS) using a 1/x weighted least-squared regression. The calibration curve was accepted if the accuracy of back-calculated concentration values at each level was less than ±15 % (±20 % at the lower limit of quantification (LLOQ)). The range was considered to be linear if the determination coefficient was greater than 0.99 and in the absence of systematic pattern in the residuals.

2.4.3. Bias, precision, uncertainty and LLOQ

To assess the precision and accuracy of the assay, QC fortified at four concentration levels (LLOQ, Low, Mid, High) of the target analytes were analyzed in six replicates and on three separate analytical series. In accordance with the WADA technical document TD2019DL [28], combined measurement uncertainty (u_c) was also assessed by quadratic combinations of the intermediate precision and the root mean square of the bias estimates. A pre-defined uncertainty acceptance criterion was set at 20 % of the mean result at each concentration level of the QCs. LLOQ was determined as the concentration at which uncertainty was below 20 %.

2.4.4. Extraction recovery and matrix effects

Extraction recovery was calculated as the ratio of the peak areas of the analyte from the pre-extracted spiked and post-extracted spiked DBS samples prepared in triplicate. Matrix effects were investigated by comparing the peak areas of the analytes in post-extracted spiked samples to peak areas of the corresponding reference standard. For each analyte, the concentration corresponding to the calibration solution 6 was used for these analyses (Supplemental Table 1).

2.4.5. Stability

DBS samples from a volunteer were collected from finger prick (20 µL onto Whatman 903™ protein saver cards), loaded into a foil pouch with a silica gel desiccant, and stored at three different storage conditions (RT, 4 °C and −20 °C) for 1 and 3 weeks. For each condition, three spots were used and extracted. The post-extraction stability of extracts was also investigated after one week of storage in the autosampler at approximately 10 °C.

2.5. Clinical samples description

2.5.1. Testosterone gel study

Fourteen healthy female volunteers participated to an open-label trial. All subjects provided written informed consent prior to any study procedures and the trial was approved by the local Ethical Committee of the Canton de Vaud in Switzerland (2018−02106, SNCTP000003264) and Swissmedic (2018DR1168), registered on www.isrctn.com (ISRCTN10122130) and was conducted in accordance with the Declaration of Helsinki as described previously [7]. Briefly, the study was divided into three 4-weeks phases corresponding to three consecutive menstrual cycles for a total of 12 weeks. Phase 1 corresponded to the control phase during which samples were collected once a week. Phase 2 corresponded to the treatment during which 0.5 g testosterone gel (Tostran® 20 mg/g) was self-administered every morning on the upper thigh and/or abdomen for 28 days. Samples were collected before the application of testosterone gel, which corresponded approximately to 24 h post-application, and with the sampling scheme similar to phase 1. Phase 3 was similar to phase 1 and corresponded to the post-treatment phase.

Serum samples were collected from antecubital vein in 8.5 mL BD Vacutainer® SST™ II Advance tubes and whole blood in 4 mL BD Vacutainer® K2EDTA tubes. DBS samples were generated either using volumetric 10-µL microsampling HemaXis DB10 kits (DBS System SA, Gland, Switzerland) from finger-prick and by depositing 20 µL of EDTA whole blood onto cards (Whatman 903™ protein saver cards), which were left to dry for a minimum of 1 h at RT and stored at 4 °C with a desiccant. Hematological variables including hematocrit (HCT) were measured in whole blood samples using a fully automated hematology analyzer (Sysmex XN-1000, Sysmex).

For the analysis of DBS generated with HemaXis DB10, two 10-µL spots were used for the quantification of steroids with the method described above. Serum samples were extracted and analyzed using a method reported earlier [27] and results were compared with those measured in the DBS and corrected with HCT level using the following equation: Corrected concentration = (DBS concentration)/(1-HCT). Comparison of the steroid concentrations obtained with the two methods was performed using the Passing-Bablok regression and correlations were calculated using Spearman's correlation method with R Studio software.

2.5.2. Comparison between finger prick and Tasso-M20 DBS

To assess the efficiency of the Tasso-M20 push-button blood collection device (Tasso Inc, WA, USA) for the measurement of the steroid profile, capillary blood was collected from fourteen healthy volunteers (seven males and seven females) using both finger-pricks and Tasso-M20 devices positioned on the upper arm. Blood samples from finger-pricks (20 µL) were deposited onto filter paper (Whatman 903™ protein saver cards), left to dry for at least 1 h, and then stored at room temperature with desiccant. The dried Tasso volumetric 20-µL tips samples were stored under ambient conditions with desiccant until analysis. The tips were analyzed as described previously for the DBS. The correlation between finger-prick DBS and Tasso-M20 DBS was calculated using Spearman's correlation method with R Studio software.

3. Results

3.1. Method validation

Quantitative performance of the method and the summary of validation results are described in Table 1(complete set of data for each QC level are shown in Table S4). Regarding selectivity, no interfering signals were observed for the ten replicates of negative blood samples at the expected retention times of the analytes, owing to the adequate chromatographic separation in conjunction with the two structure specific MRM transitions.

The linearity range was determined for each compound and the accuracies of the back-calculated concentrations at each calibration level met the predefined criteria (less than ±15 % and less than ±20 % bias at LLOQ). The method demonstrated satisfactory accuracy, expressed in terms of bias, and precision at each QC level (Table S4 at each QC concentration). The relative bias did not exceed 10 % for any QCs. The precision, represented by repeatability and intermediate precision, was below 10 % with the exception of the lowest QC for glucuro-conjugated androsterone and eti-

Table 1
Summary of validation results. RT: retention time; LLOQ: lower limit of quantification.

Analyte	RT (min)	Bias (%)	Repeatability (%)	Intermediate Precision (%)	Combined Uncertainty (%)	Linearity range (ng/mL)	LLOQ (pg/mL)	Recovery (%)	Matrix effect (%)
Testosterone	9.81	2.6–3.9	2.1–3.3	2.1–4.6	3.6–6.5	0.02–25	20	110	95
Epitestosterone	11.5	2.4–3.8	1.5–3.2	2.6–3.2	4.1–5.2	0.05–10	50	110	99
Androstenedione	8.74	2.5–6.2	1.3–2.3	1.6–3.6	3.5–7.3	0.1–25	100	108	93
DHEA	10.61	2.1–3.6	2.3–2.9	2.6–3.9	3.7–5.7	2.5–150	2500	111	92
DHT	12.04	1.9–2.6	1.8–2.8	1.9–2.8	3.4–4.1	0.25–10	250	115	91
Progesterone	13.42	2.1–4.7	2.1–2.8	2.1–3.2	3.1–6.0	0.025–25	25	112	93
17α-hydroxyprogesterone	10.42	2.2–3.7	1.7–3.1	2.2–3.9	3.4–5.4	0.1–25	100	112	97
Cortisol	5.07	3.5–5.2	1.5–5.0	1.6–5.5	4.7–9.0	1–400	1000	110	97
Corticosterone	6.97	3.5–4.6	1.5–4.9	1.8–4.9	4.7–7.5	0.25–100	250	112	92
Deoxycorticosterone	9.45	2.6–5.9	1.9–2.9	2.4–4.1	4.4–6.6	0.1–10	100	112	92
11-Deoxycortisol	7.36	2.2–3.6	2.2–3.0	2.2–4.5	3.6–6.1	0.1–5	100	110	90
Androsterone Glucuronide	9.61	3.7–8.6	3.6–7.2	4.8–11.8	6.5–16.0	1–100	1000	67	100
Etiocholanolone Glucuronide	8.99	3.4–9.6	4.8–13.3	4.8–14.1	6.7–20.4	1–100	1000	74	105
Testosterone Sulfate	5.29	2.8–3.4	2.6–3.6	3.5–3.9	5.0–5.8	0.25–25	250	84	96
Androsterone Sulfate	8.18	2.4–5.6	2.1–3.0	2.8–3.4	4.2–6.9	5–2500	5000	82	95
Etiocholanolone Sulfate	7.89	2.7–4.8	1.9–3.7	1.9–5.8	4.5–7.8	5–2500	5000	84	90
DHEA Sulfate	5.95	2.7–6.3	2.3–5.0	2.9–5.8	4.4–8.3	50–10000	50000	82	96
Dehydroandrosterone Sulfate	6.92	2.4–7.3	2.5–6.1	3.0–6.4	4.4–11.0	0.25–100	250	80	95
Epiandrosterone Sulfate	6.7	4.4–9.4	3.9–8.2	5.0–8.2	7.6–13.9	0.5–2500	5000	80	96

ocholanolone. Nevertheless, at this concentration (1 ng/mL), the precision was considered acceptable as the results were still below 15 %. Combined uncertainty, representing statistical dispersion of the values attributed to a measured quantity, was also assessed in agreement with WADA regulations (TD2019DL), and the estimates were all below 20 % as a predefined uncertainty acceptance criterion except for the lowest QC of etiocholanolone glucuronide with an uncertainty estimate of 20.4 %.

Recovery was excellent for all unconjugated steroids (108–115 %), while it demonstrated lower but still acceptable results for sulfate (80–84 %) and glucuronide (67–74 %) conjugated metabolites. No significant matrix effect was observed for any analyte. The carryover was negligible with all traces below 0.2 %.

Concerning the stability of the analytes in DBS, no significant difference was observed between the storage conditions (RT, 4 °C and −20 °C) for any steroid, except for progesterone. After 3 weeks of storage, progesterone level was significantly lower when stored at −20 °C compared to RT (data not shown). Extracts stored in the autosampler for one week demonstrated similar results to fresh extracts.

3.2. Analysis of DBS from clinical study

To validate the use of DBS in clinical setting and physiological condition, the hematocrit-adjusted concentration measured in DBS during the control phase (phase 1) of the clinical study was compared with those measured in serum for each steroid. Epitestosterone, DHT, deoxycorticosterone, 11-deoxycortisol and testosterone sulfate were below LLOQ or could not be detected and were thus not included for the further comparison. Where the reference could be made to serum concentrations, Passing-Bablok regression was assessed for each compound and the plots are presented in Fig. 1. The slope, intercept and their confidence intervals of 95 % (95 % CI) as well as the correlation coefficient were evaluated. All compounds demonstrated a correlation coefficient higher than 0.8 and a slope between 0.69 and 1.13. Exempting testosterone, androstenedione, cortisol, androsterone sulfate and etiocholanolone glucuronide, a positive intercept was obtained for steroid concentrations, suggesting a slight overestimation in DBS.

To further evaluate the use of DBS in the doping context for the detection of testosterone doping, the samples collected during the treatment period (phase 2) and after (phase 3) were also analyzed. To avoid any potential interference from testosterone residue on the fingers used for gel application, DBS samples generated with EDTA whole blood were also included and analyzed. Testosterone concentration in capillary DBS exhibited unsatisfactory values in comparison to serum with extremely high values measured for most samples during and after T gel administration (Fig. 2A), while testosterone concentration measured in DBS generated using whole blood collected in EDTA tubes highly correlated with testosterone concentration in serum (Fig. 2B). Notably, both matrices demonstrated a similar trend when mean concentrations were plotted longitudinally over the three study phases (Fig. 2C). The other compounds demonstrated similar results to phase 1 alone (Fig. S1).

3.3. Comparison between finger prick and Tasso device DBS

Fourteen individuals were recruited for the comparison between regular finger-prick or Tasso-M20 push-button device for the analysis of steroid concentrations in DBS. All quantified steroids demonstrated strong correlation between both collection methods. The results are presented for six free steroids (Fig. 3) and the comparison for the other compounds are available in Fig. S2.

4. Discussion

The method described in this study demonstrated reliable quantitative performance for the profiling of a panel of steroids in DBS. As the chromatographic separation and MS/MS conditions used in this study were already optimized for the analysis of serum samples [27], the emphasis was put on the processing of DBS samples. Based on validation data, combined uncertainty was assessed for each analyte in compliance with the WADA requirements for quantitative methods, consisting of the intermediate precision and bias components. For most compounds, uncertainty was estimated below the predefined acceptance criterion of ±20 %. In the case of etiocholanolone glucuronide, the combined uncertainty was 20.4 %

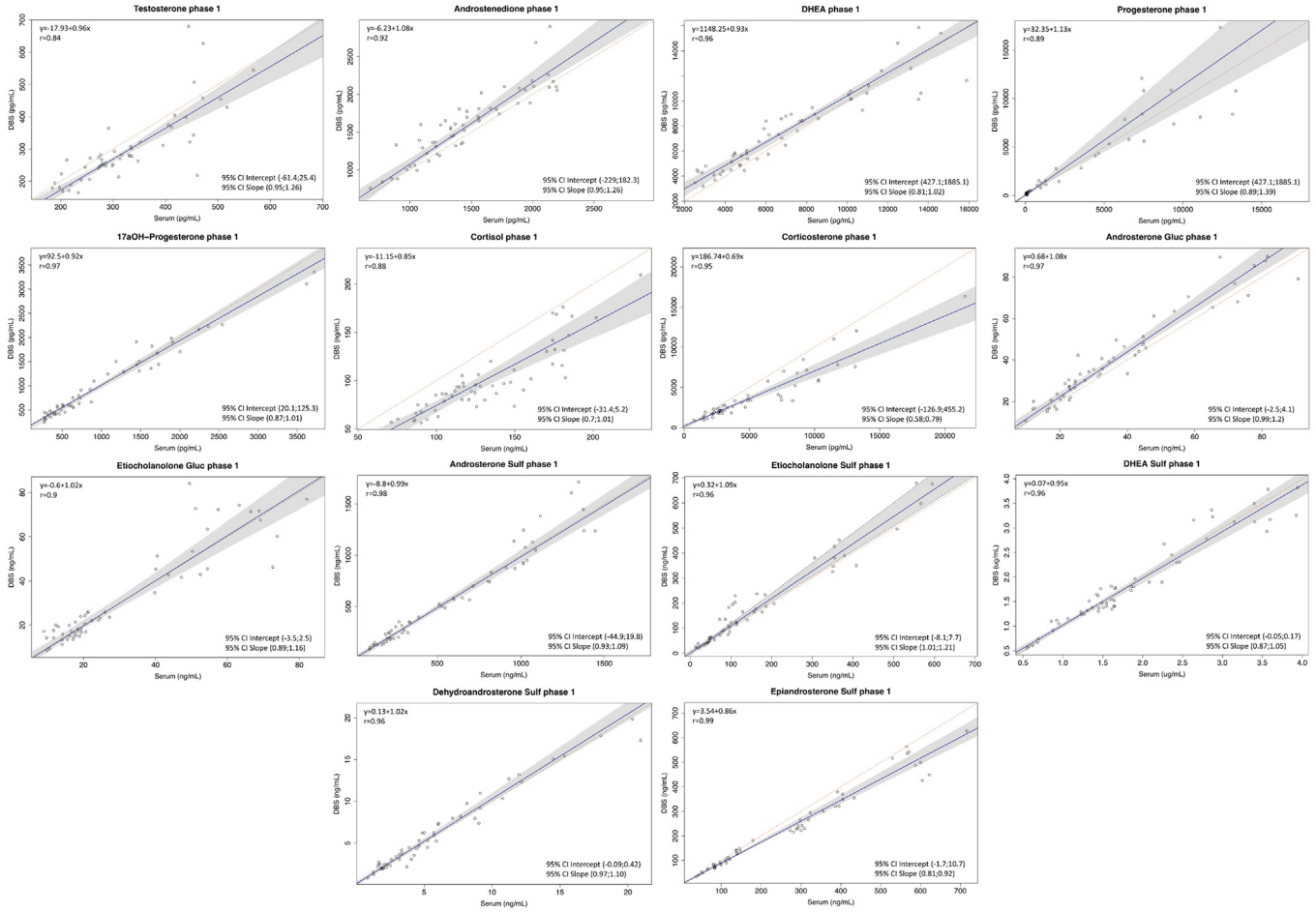

Fig. 1. Passing-Bablok regression plots for steroids quantification in serum vs DBS collected during phase 1. Blue line indicates regression line, red dashed line indicates identity line, and the confidence bands for regression are delimitated in grey. R is the correlation coefficient and 95 % CI corresponds to the 95 % confidence interval.

at the lowest QC level, which could be related to the low ionization efficiency of glucuronide-conjugated species in the negative mode. Nevertheless, as the result was still very close to the predefined acceptance criterion, it was considered fit for purpose.

The major challenge when developing a DBS method for steroid analysis is to reach a sufficient sensitivity for the target compounds that may be present in low circulating levels, especially in women, and to cover large and various ranges of concentration. The sensitivity of this developed method was satisfactory for the majority of the critical compounds, such as testosterone or progesterone (Fig. S3). DHT, a potential biomarker of testosterone administration, was the only compound for which the current sensitivity (LLOQ at 250 pg/mL) may be inadequate for the analysis of DBS samples of female athletes, which is in part due to its mass spectrometric properties. Furthermore, slightly higher uncertainty estimations were obtained at low concentration for the glucuro-conjugated species, but as they generally circulate at high concentrations in blood, no true sensitivity issues were encountered [29]. Finally, the concentration of the steroids panel remained generally stable when stored for 1–3 weeks either at RT, 4 °C or −20 °C. It highlights that, contrary to serum samples usually used for the steroid profiling, DBS cards can be transported and/or stored at RT with a minimal risk of degradation.

To first evaluate the applicability of the developed method in physiological condition, capillary DBS collected over 4 weeks during a clinical study involving healthy eumenorrheic women were analyzed and concentration values were compared to corresponding serum samples. For most compounds, satisfactory correlation ($r>0.84$) was discovered between DBS and serum. Passing-Bablok regression based on robust, non-parametric model was used to evaluate analytical methods agreement. The model is based on the hypothesis that if the 95 % CI for intercept includes value zero and 95 % CI for slope includes value 1, there is no constant (intercept) nor proportional (slope) difference between the two methods. These criteria were met for testosterone, androstenedione, cortisol and all conjugated metabolites (with the exception of epiandrosterone sulfate). For DHEA, 17α−OH-progesterone and progesterone, 95 % CI for intercepts did not include 0, indicating a constant difference between the two methods. It is mainly due to higher values measured in DBS at low concentration for these compounds, which could be partly explained by better recovery from DBS. For epiandrosterone sulfate, although upper limit of 95 % CI for slope was close to 1 (0.92), the two methods had a slight proportional difference with higher concentrations measured in serum. Corticosterone also demonstrated a proportional between both methods with consistent higher values in serum suggesting that both methods should not be used simultaneously. Overall, we can conclude that results obtained from both methods were in high agreement and that measured concentrations in DBS can be compared with those of serum for most compounds. These results also emphasize the necessity to adjust concentration measured in DBS with HCT [30]. Furthermore, these samples were stored for more than one year with desiccant before their analysis underlining the analytes' stability in DBS.

Longitudinal monitoring of testosterone in serum has been proposed as powerful approach for the detection of testosterone

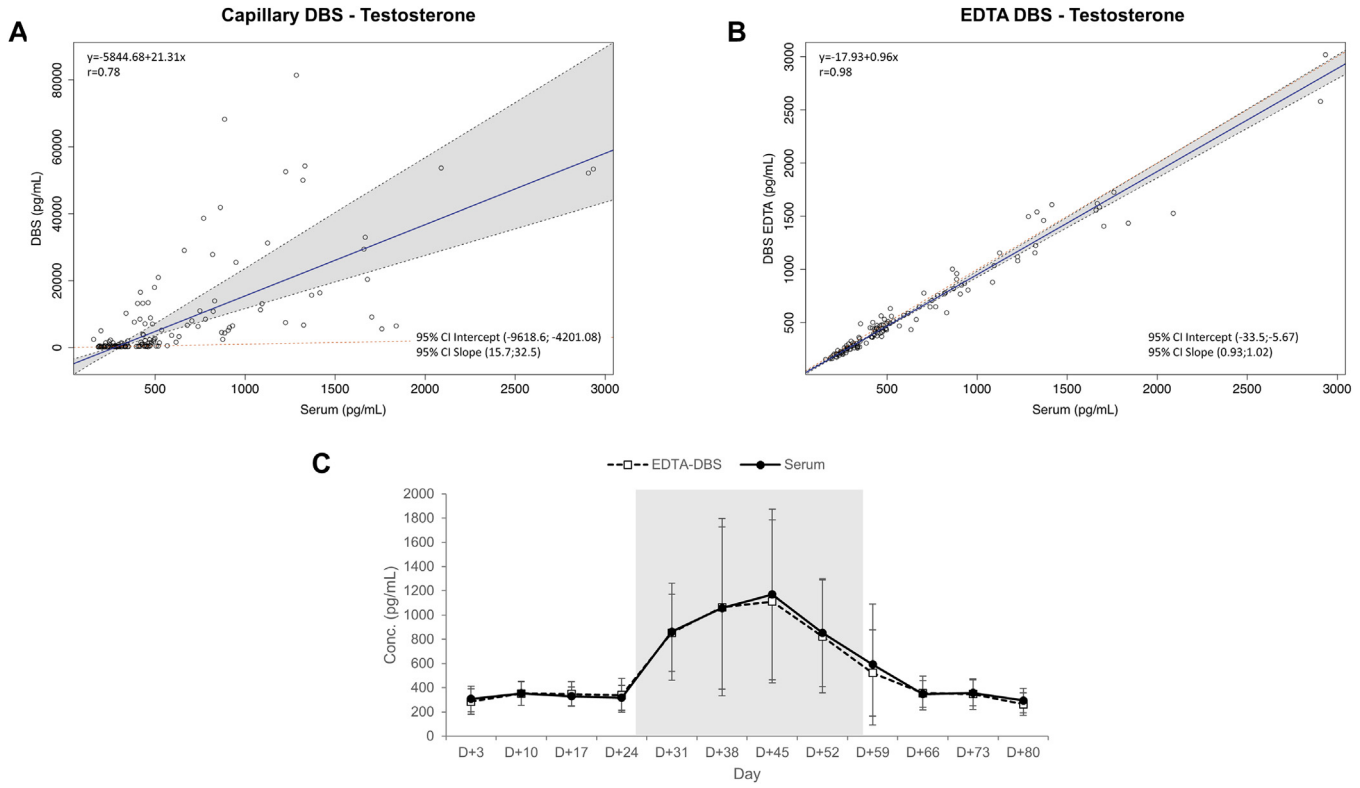

Fig. 2. Passing-Bablok regression plots of testosterone concentration values between serum and (A) capillary DBS and (B) EDTA-DBS during the whole study. Blue line indicates regression line, red dashed line indicate identity line, and the confidence bands for regression are delimitated in grey. R is the correlation coefficient and 95 % CI corresponds to the 95 % confidence interval. (C) Mean plot of testosterone concentration measured in EDTA-DBS and serum throughout the study. The grey area corresponds to the treatment phase and divides the three study phases.

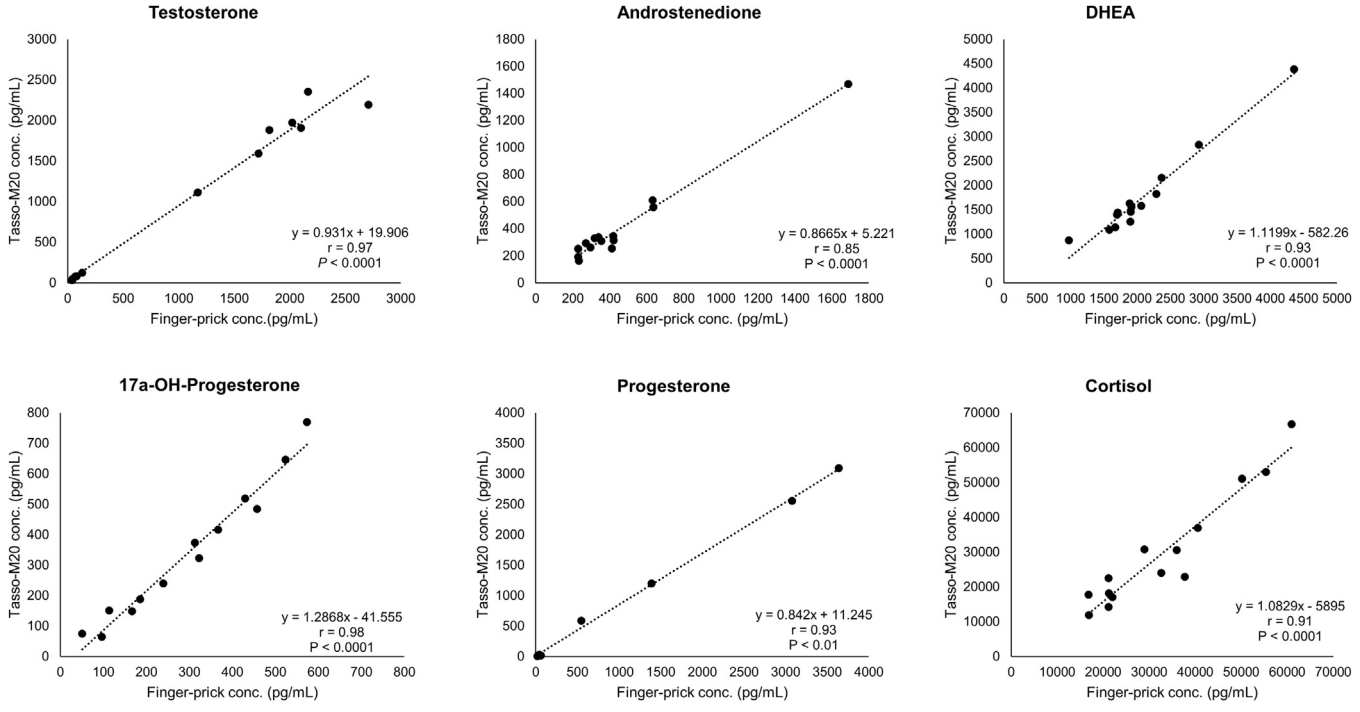

Fig. 3. Correlations between concentration levels of six free steroids in finger-prick vs Tasso-M20 DBS samples.

administration in women [7]. Therefore, to evaluate the applicability of DBS in the context of anti-doping, DBS samples from the two following phases (testosterone gel administration – phase 2 and wash-out – phase 3) of the clinical study were also processed. Related to the use of topical testosterone formulation, a previous study showed that considerable amounts of testosterone (60 % after 8 h) remained on the intact skin for several hours after testosterone gel application and evaporation of the alcohol

vehicle [31]. Thus, to avoid any potential interferences from testosterone residue on fingers used for gel application, DBS samples generated with EDTA tubes collected at the same time and representing rather systemic testosterone concentration were also analyzed with the developed method. While testosterone levels demonstrated high concordance between EDTA-DBS and serum, capillary DBS displayed some extreme values during and after testosterone administration. It seemed that the testosterone values increased as the treatment progressed suggesting a potential accumulation. Indeed, following its application, the gel dried and the steroid is absorbed into the stratum corneum, which acts as a reservoir before slowly releasing testosterone into the circulation [32]. On the contrary, mean testosterone concentration exhibited similar results between EDTA-DBS and serum when data were longitudinally plotted. Furthermore, the other analytes concentrations demonstrated satisfactory correlation with the serum matrix during and after the treatment period. These findings confirm the hypothesis that testosterone is indeed persisting in the finger skin, and that the local residual testosterone could interfere and generate high concentrations in capillary blood when collected with finger prick. This observation could also partly explain the low bioavailability of testosterone gel formulation. Although this hypothesis is highly likely, we emphasize that the risk of interpersonal contamination is very low as reported in [31], especially if the site of application is covered or washed [33]. We point out that the alcoholic testosterone gel, which is in contact with the application hand, remains in the finger skin and generates only locally high capillary testosterone concentration. Therefore, a high capillary testosterone concentration in DBS is highly likely to reflect a direct contact with exogenous testosterone gel. Nevertheless, further studies should be carried out to confirm this hypothesis by combining testosterone administration and multiple capillary blood collection sites.

To overcome this potential pitfall, the Tasso-M20 push button device was also evaluated as alternative collection method. This device allows collecting capillary blood on the upper arm using volumetric absorptive microsampling (Fig. S4). We demonstrated that steroid concentrations were comparable between finger-prick DBS and Tasso DBS collected from the fingers and arms of fourteen individuals, thereby demonstrating the independence from spot support and sample collection site. While this approach might be beneficial in this context, it could also generate similar issues if testosterone gel is administered on the upper arm, at the same location as the Tasso-M20.

5. Conclusion

In summary, a fit-for-purpose UHPLC-MS/MS method was developed and validated for the quantification of a panel of steroids in DBS. This method could be applied to anti-doping as a complementary approach for the longitudinal monitoring of steroid profile and detection of testosterone administration in the ABP allowing for more frequent sampling and for targeting blood (serum) and urine sample collection that would be used for a full steroid profile and for confirmatory GC/C/IRMS analysis. The increased sampling frequency would provide a better estimation of natural baseline variability of a given athlete and would provide a better resolution of a possible doping picture [34]. This approach could also be employed for the monitoring of steroid-related pathologies in the clinical context. Indeed, for patients requiring regular medical visits or for whom venipuncture is complicated (neonates, elderly patients), DBS collected with Tasso-M20 or finger prick with HemaXis DB10 at home or on-site could be a valuable alternative to classical serum collection.

CRediT authorship contribution statement

Olivier Salamin: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing - original draft. **Raul Nicoli:** Methodology, Validation, Writing - review & editing. **Cheng Xu:** Investigation, Writing - review & editing. **Julien Boccard:** Validation, Writing - review & editing. **Serge Rudaz:** Validation, Writing - review & editing. **Nelly Pitteloud:** Resources, Supervision, Project administration. **Martial Saugy:** Supervision, Project administration, Funding acquisition. **Tiia Kuuranne:** Resources, Supervision, Funding acquisition, Writing - review & editing.

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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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114280.

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