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High-resolution mass spectrometry as an alternative detection method to tandem mass spectrometry for the analysis of endogenous steroids in serum

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ABSTRACT

Recently, steroid hormones quantification in blood showed a promising ability to detect testosterone doping and interesting complementarities with the urinary module of the Athlete Biological Passport (ABP). In this work, an ultra-high pressure liquid chromatography–high-resolution mass spectrometry (UHPLC-HRMS) method was developed for the quantification of eleven endogenous steroids in serum. The performance of the full scan and targeted SIM acquisition modes was evaluated and compared to the performance of tandem mass spectrometry (MS/MS). Passing-Bablok regressions and Bland-Altman plots were assessed for each analyte of interest, and concentration values measured by HRMS showed high correlation with the ones obtained by MS/MS for all target hormones, with low absolute differences in the majority of cases. A slight decrease in terms of sensitivity was observed with HRMS in both acquisition modes, but performing an analysis of variance multiblock orthogonal partial least squares (AMOPLS) on the dataset obtained with all three methods revealed that only 0.8% of the total variance was related to instrumentation and acquisition methods. Moreover, the evaluation of the testosterone administration effect over time highlighted testosterone itself and dihydrotestosterone as the most promising biomarkers of exogenous testosterone administration. This conclusion suggests that HRMS could provide suitable performance for blood steroid analysis in the anti-doping field.

1. Introduction

The detection of testosterone (T) abuse in sport is currently achieved through the steroidal module of the Athlete Biological Passport (ABP). The so-called "steroid profile" was implemented in 2014, and it is obtained from urinary concentrations of six endogenous anabolic androgenic steroids (EAAS) measured by gas chromatography-(tandem) mass spectrometry (GC–MS(/MS)). It consists of longitudinal monitoring, with individual reference intervals calculated by a Bayesian adaptive model, of five ratios known to be potentially altered following the administration of synthetic forms of EAAS: androsterone/testosterone (A/T), androsterone/etiocholanolone (A/Etio), 5α-androstane-3α,17β-diol/5β-androstane-3α,17β-diol (5αAdiol/5βAdiol), 5α-androstane-3α,17β-diol/Epitestosterone (5αAdiol/E) and the most important, testosterone/epitestosterone (T/E) [1]. Although this new approach clearly improved detection capabilities in comparison with the previous population threshold criterion of T/E > 4, it still suffers from major drawbacks. These include the large presence in the urine matrix of both endogenous (e.g., metabolism, ethnicity) and exogenous (e.g., ethanol, bacterial contamination) confounding factors, which can influence either the quantification of the urinary steroid profile or its interpretation [2], and the difficulty of detecting doping in individuals with a genetic polymorphism for UGT2B17 enzyme activity [3–6] and/or in athletes who have been administered T gel and patch formulations [7].

In the last few years, several studies have been conducted with the aim of improving steroid abuse detection capabilities. At first,

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major efforts were focused on the identification, by means of GC–MS(/MS) platforms, of new urinary biomarkers of EAAS abuse to be added to the panel of ratios included in the steroid profile [8–11]. Then, research broadened and oriented to UHPLC–MS/MS methods as well, as this type of technique is faster, more sensitive and capable of detecting phase II metabolites of steroid hormones in their intact form [12–14], unlike GC–MS analysis which always requires a derivatization step, eventually preceded by a hydrolysis step. More recently, high-resolution mass spectrometry (HRMS) has also been used to provide innovation in the context of steroid analysis. Indeed, holistic approaches such as metabolomics (defined as steroidomics [15]), allowed the untargeted evaluation of a large number of compounds, thus also representing a promising strategy for the discovery of new biomarkers and metabolites for anti-doping purposes [16,17]. Nevertheless, the use of HRMS platforms by anti-doping laboratories for screening analyses is constantly increasing [18–21], even if their use for quantification purposes is still not widespread.

In the anti-doping field, both endogenous and exogenous steroids have been traditionally analyzed in urine, but a first attempt of endogenous steroid analysis in blood was recently conducted [22]. The blood matrix is clearly more difficult to manipulate/contaminate than urine, and quantitative analysis of target compounds in blood represents a real snapshot of athlete physiological status, a key aspect for possibly better discriminating doping from pathologies. In addition, the study of steroid metabolism in blood could also be particularly relevant to explain diseases associated with a possible malfunction in steroidogenesis, in particular steroid metabolism enzymes (e.g. congenital adrenal hyperplasia, CAH) as well as in environmental sciences to better understand the mode of action of endocrine disrupting chemicals. On the other hand, the blood matrix also raises some concerns in the context of sports drug testing: its sampling is more invasive compared to urine, the collected volume is significantly lower and more accurate conditions for transportation and storage are needed.

In this work, a UHPLC-HRMS method was developed for the quantification of 11 endogenous steroid hormones in serum, including major androgens, progestogens and corticoids. The performance of the full scan (FS) and targeted selected ion monitoring (t-SIM) acquisition modes was evaluated and compared to that of UHPLC–MS/MS obtained in a previous work using samples from a testosterone clinical study [22]. Furthermore, in addition to quantitative performance, the ability to describe the exogenous testosterone administration by the three acquisition methods was also evaluated by means of an ANOVA-based multivariate statistical analysis.

2. Materials and methods

2.1. Study samples

Serum samples used for the method comparison were obtained from a T administration clinical trial (Swissmedic protocol n° 155/11) with 19 healthy male volunteers, who were administered twice with a T transdermal patch (Testopatch® 2.4 mg/24 h, Pierre Fabre Pharma GMBH, Freiburg, Germany) followed by T undecanoate capsules (Andriol Testocaps®, Essex Chemie AG, Luzern, Switzerland). The detailed description of the study is presented elsewhere [7].

2.2. Chemicals and reagents

Available standards of endogenous steroids were purchased from Lipomed (Arlesheim, Switzerland), Cerilliant (Round Rock, TX, USA), Steraloids (Newport, RI, USA) and Sigma-Aldrich (Buchs, Switzerland), while all labeled internal standards (IS) were provided by the Australian Government National Measurement Institute (Pymble, Australia). Methanol (MeOH) was purchased from Macron Fine Chemicals (Deventer, Netherlands), and acetonitrile (ACN) ULC/MS (>99%) and formic acid (FA) ULC/MS (99%) were supplied by Biosolve BV (Valkenswaard, Netherlands). Charcoal-dextran stripped human serum was obtained from Dunn Labortechnik GmbH (Asbach, Germany). Deionized water was obtained by a Milli-Q®-grade system (Millipore, USA) and was used for the preparation of all buffers and solutions.

Analytes and IS mixture solutions were prepared in MeOH at appropriate concentrations and used for the preparation of calibration samples in depleted serum. Linear calibration curves were created for each analyte (weighting 1/x) to measure steroid concentration, and calibration samples were used for the extraction of lower limits of quantification (LLOQ) and accuracy values of the method. Details concerning the composition and concentration of the calibration samples and IS mixtures are given in Supplementary Material Tables S1 and S2.

2.3. Sample preparation and UHPLC conditions

All the details concerning the extraction protocol and chromatographic conditions have been described in [22]. Briefly, a supported liquid extraction SLE+ 400 μL (Biotage, Uppsala, Sweden) in a 96 well-plate format was used to extract steroid hormones. Each serum sample (200 μL) was spiked with 20 μL of the IS mixture, diluted with 200 μL of water and then agitated for few minutes. Then, 400 μL of sample was loaded on each well and positive pressure of 3 psi was applied for 30 s to facilitate sample adsorption; after 5 min, the elution was carried out by adding 700 μL of DCM to each well and applying a pressure of 3 psi for 1 min. After evaporation, 10 μL of reconstituted extracts were injected on a UPLC chromatographic system (Waters, Milford, MA, USA) equipped with an Ethylene Bridged Hybrid (BEH) C_{18} column (100 × 2.1 mm, 1.7 μm; Waters) set at 30 °C and a pre-column. The mobile phases consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in ACN, and the flow rate was set at 400 μL/min. The gradient started linearly from 2% to 25% B over 0.5 min, followed by an increase to 58% B over 5.5 min and by a further increase to 98% B over 2 min; the column was then re-equilibrated for 3 min at initial conditions.

2.4. MS conditions

2.4.1. MS/MS analysis

The UPLC system was coupled to a Xevo-TQ S triple quadrupole MS/MS system from Waters working in ESI positive and selected reaction monitoring (SRM) mode. The detailed instrumental UHPLC–MS/MS conditions (SRM transitions, ESI conditions, cone voltages and collision energies) are described in a previous article [22].

2.4.2. HRMS analysis

HRMS analyses were performed by coupling the UPLC system to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Mass calibration (< 3 ppm) was performed once a week using the Pierce® LTQ Velos ESI Positive Ion Calibration standard mixture (Thermo Fisher Scientific) containing *n*-butylamine, caffeine, MRFA (peptide of Met-Arg-Phe-Ala acetate salt) and Ultramark 1621. Detection of the targeted steroids was performed in positive ESI in both FS and t-SIM acquisition modes. The heated ESI source (HESI II) was used with a probe heater temperature of 425 °C, and the sheath gas and auxiliary gas pressures were set to 50 and 15 arbitrary units, respectively. The sweep gas flow was set to 3 arbitrary units. The ion spray voltage was set to 4.5 kV, the capillary temperature to 250 °C and the S-Lens RF level