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Comprehensive steroid profiling by liquid chromatography coupled to high resolution mass spectrometry

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ABSTRACT

A steroidomics workflow has been developed in the objective of monitoring a wide range (n > 150) of steroids in urine. The proposed workflow relies on the optimization of an adequate SPE extraction step followed by an UHPLC-HRMS/MS simultaneous analysis of both free and conjugated forms of C18, C19 and C21 steroid hormones. On the basis of 44 selected steroids, representative of main classes of steroids constituting the steroidome, the performances of the developed workflow were evaluated in terms of selectivity, repeatability (< 13%) and linearity (R2 > 0.985 in the concentration range [0.01–10 ng/mL]). As metabolites identification and characterization constitute the bottleneck of such profiling approaches, a homemade database was created encompassing a large number of characterized free and conjugated steroids (n > 150) for putative steroid-like biomarkers identification purposes. The efficiency of the workflow in highlighting fine modifications within the urinary steroidome was assessed in the frame of an anabolic treatment involving an intra-muscular administration of boldenone undecylenate (2 mg/kg) to veals (n = 6) and the investigation of potential steroid biomarkers. Besides monitoring known phase II metabolites of boldenone in the bovine specie, namely, boldenone glucuronide and sulfate, the applied strategy also permitted to observe, upon boldenone administration, a modified profile of epiboldenone glucuronide. Furthermore, 31 signals corresponding to non-identified steroid species could also be highlighted as impacted upon the exogenous steroid treatment. This study is the first to simultaneously investigate both free and conjugated C18, C19 and C21 steroid hormones in their native form using UHPLC-HRMS/MS and allowing their comprehensive profiling. This strategy was probed in-vivo.

1. Introduction

Steroid hormones are non-polar compounds regulating the metabolism via the endocrine system. They are essential to physiological activities, such as metabolic homeostasis, physical development, and sexual maturation; therefore, their study has been gathering the interest of the scientific community, in diverse fields, for more than 70 years [1]. For instance, in clinical studies, steroid hormone measurement can help with diagnosing endocrine disorders, such as con-genital adrenal hyperplasia (CAH), infertility and cancers [2–8]. Therefore, considering the importance of these com-pounds in human health, reliable and precise analytical methods, also offering high sensitivity, are increasingly required for the qualitative and quantitative determination of these compounds. Quantification of steroid hormones in patients through robust analytical strategies is therefore considered as promising for early disease diagnosis. Recently the global analysis of metabolites through metabolomics approaches was performed in different fields enabling discovering new biomarkers. Such methodology was also applied to the analysis of steroids leading to the cur-rent trend observed in steroid analysis consisting in a shift from few compounds measurement to the determination of a large panel of steroids [9,10]. Such strategy is usually referred to as steroid profiling or steroidomics and has been applied in different areas. The overall evaluation of steroid perturbations has thus recently been successfully investigated in a context of public health concerns related to the increasing levels of endocrine disrupting chemicals (EDCs) found in the environment [11]. Jeanneret et al. demonstrated biochemical alterations occurring in human urinary steroidome as a result of dioxin exposure [12]. In the human or horseracing anti-doping arena, profiling steroids is also essential. It enables detecting potential misuses with naturally occurring steroids, as it is proven that administration of these steroids induces qualitative and quantitative modifications in the global steroid profile [13–23]. In this context, monitoring a range of modified endogenous steroid concentrations allows screening for hormone abuse, providing, as reference purposes, the establishment of “undisrupted” steroid profiles [24]. Since the endogenous steroid profile is known to be affected

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upon exogenous steroid administration, such steroidomic-based analytical strategy has also proven its efficiency for chemical food safety purposes, in the context of controlling the misuse of growth promoters in livestock [25–27].

First steroidomics studies were based on selective extraction and analysis of limited set of endogenous steroids from the biofluids of interest, mainly urine and blood, through specific and extensive protocols involving several SPEs purification and liquid-liquid extraction steps before final derivatisation prior to GC–MS [24] or LC–MS [10,28]analyses. Besides such targeted approach, steroidomic studies may also be implemented through untargeted analysis of the steroidome, or strategies combining both targeted and untargeted analysis [29,30]. The implementation of this approach presents however an analytical challenge in terms of steroid extraction and monitoring with regard to the range of polarity exhibited by these compounds and the need to attain a certain level of selectivity, sensitivity and analytical robustness. For instance, urine is a complex biological matrix which contains a large panel of steroid hormones (e.g. C21 pregnanes, C21 corticosteroids, C19 androgens, C18 estrogens) that are present under their free or conjugated (e.g. glucuronide, sulfate) forms, and it is now well established that relevant and complementary information is provided by the analysis of both forms, allowing for a deeper insight into the metabolic variations [22]. Hence, a careful choice of the extraction, separation, detection and processing procedures should be adopted to allow a comprehensive overview of steroidome disruptions. Unlike gold standard GC–MS approaches, LC–MS techniques allow combining both free and conjugated steroids intact forms analysis [31,32], offering thus the possibility to profile a larger panel of steroid compounds and access to higher biological information. Such LC–MS strategy permits also to avoid the generation of possible artifacts due to the GC–MS derivatization step [33,34]. Therefore, LC–MS techniques have been spreading among laboratories for that specific application. Triple quadrupole instruments (QqQ) still represent the preferred MS platform for quantification, but instruments offering simultaneous qualitative and quantitative possibilities using high resolution MS such as Orbitrap or Time-of-Flight (TOF) are now presenting performances in terms of quantification approaching QqQ instruments [10,22,35–37]. In parallel of these analytical developments, chemometrics have become mandatory to deal with the huge amount of data generated using such approaches.

The aim and originality of the present work was to develop a comprehensive urinary steroidomics approach based on a targeted and non-targeted monitoring of a large number of C18, C19 and C21 steroids, both under their free and glucurono- / sulfo-conjugated forms using UHPLC-HRMS/MS analysis, with the objective of proposing an analytical strategy enabling monitoring the largest number (> 150) of possible steroids in a single run, offering therefore a global picture of the steroidome. The sample preparation was optimized assessing different SPE cartridges and conditions, which have been compared in terms of extraction recovery. The chromatographic separation and the ion source transfer efficiency were also optimised in terms of broadening the steroid polarity range detection with higher sensitivity and selectivity. As a proof of concept, the developed method was applied in the frame of an animal experiment involving the intramuscular administration of boldenone undecylenate (2 mg/kg) to six veals in order to identify putative biomarkers of such anabolic practice in a chemical food safety context.

2. Experimental section

2.1. Chemicals

All solvents used in this study were whether of LC–MS grade (acetonitrile, water, methanol, formic and acetic acid) or HPLC grade (ethyl acetate) and were purchased from Sigma Aldrich Chromasolv Reagents (St. Louis, MO, USA) and VWR International (Pessac, France). Ammonium acetate anhydrous powder was purchased from Merck KGaA, (Darmstadt, Germany). Two ammonium acetate solutions (pH = 7.2) at a concentration of 250 and 50 mM were prepared by dissolving respectively 19.27 and 3.85 g of ammonium acetate in 1 L of LC–MS grade water. Two formic acid solutions at 5 and 1% were also prepared by diluting respectively 50 and 10 mL of LC–MS grade formic acid in 1 L of LC–MS grade water. SPE Isolute C18 and Oasis HLB cartridges of 1 g were pur-chased respectively from Biotage (Hengoed, UK) and W-ters (Milford, MA, US). MSCAL5 ProteoMassT LTQ/FT-Hybrid ESI Pos/Neg (Sigma–Aldrich) (Calmix-positive, for the positive-ionization mode, consisting of caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate, and Ultramark 1621; Calmix-negative, for the negative-ionization mode, consisting of same mixture plus sodium dodecyl sulfate and sodium taurocholate) was used for the external calibration of the MS instrument.

2.2. Stock solutions

All reference compounds (n = 152) were purchased from Steraloids (Newport, RI, USA), Sigma Aldrich (St. Louis, MO, USA), or National Measurement Institute (NMI, Pymble, Australia). All compounds were prepared at a concentration of 1 mg/mL in several solvents according to their physico-chemical properties and divided in mixtures as detailed in Supporting Information. Each mixture was then split in HPLC vials and subsequently evaporated under a gentle nitrogen stream at room temperature. These reference mixtures were finally stored at −80 °C to serve as reference during the whole study.

2.3. Biological material

The animal experiment was approved by the French Committee of Ethics (Protocol Nº 02,323.01) and conducted within Oniris (France) facilities. After 1 month of acclimatization, 6 veals (Prim'Holstein), aged 4 months, were treated by intramuscular (i.m.) injection of boldenone undecylenate (2 mg/kg body weight). Animals were daily fed with 2.7 kg/animal of pelletized food (Claris Démarrage G, France). Water and hay were also available ad libitum. Urine samples were collected, at the same moment of the day, in the morning before the animals were fed, over the four days (n = 4) preceeding boldenone administration and on days 1, 2, 3, 4, 5, 7, 9, 12, 15, 18, 22, 28 and 35 following the administration (n = 13). Subsequently, all urine samples were divided in aliquots (V = 5 mL) and stored at −20C° before analysis. An aliquot of urine samples collected before administration (n = 4 days) were pooled to obtain a Reference Urine which was used for method development optimization only.

2.4. Sample preparation

A Quality Control (QC) sample was prepared by pooling 500 µL of each urine sample (collected both before and after administration), previously thawed on ice. Afterwards, the different samples and QC were normalized through water dilution according to their specific gravity (SG) (Jacob, 2014), which was measured by refractometry, in order to obtain a final reference specific gravity (SGref) of 1.007 for each urine sample. Four mL of each normalized urine sample, buffered with 1 mL of ammonium acetate solution at 250 mM, were then loaded onto C18 cartridges, previously conditioned with 5 mL of methanol and 5 mL of aqueous solution of ammonium acetate at 50 mM. After loading the sample, the cartridges were first washed with 5 mL of a mixture of solution of ammonium acetate (50 mM)/methanol (60:40,v:v) and then with 5 mL of a mixture water/methanol (95:5, v:v). A first elution was performed by 5 mL of a mixture of methanol/water (90:10,v:v), followed by a second and third elutions using the same collecting tube by, respectively, 2 mL of methanol and 3 mL of a mixture ethyl acetate/methanol (70:30,v:v). The eluate was then evaporated under gentle nitrogen stream at 45 °C and reconstituted in 100 µL of a mixture methanol/water (50:50,v:v) before transfer in auto-sampler vials for UHPLC-HRMS analysis.