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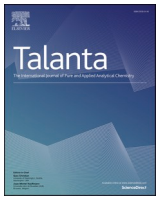

High throughput LC-MS/MS method for steroid hormone analysis in rat liver and plasma – unraveling methodological challenges

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ABSTRACT

Comprehensive reference data for steroid hormones are lacking in rat models, particularly for early developmental stages and unconventional matrices as the liver. Therefore, we developed and validated an enzymatic, solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify a panel of 23 steroid hormones in liver and plasma from adult and neonatal rats. Our approach tackles methodological challenges, focusing on undesired byproducts associated with specific enzymatic treatment, and enables a thorough assessment of potential interferences in complex matrices by utilizing unstripped plasma and liver. We propose an optimized enzymatic hydrolysis protocol using a recombinant β-glucuronidase/sulfatase mix (BGS mix) to efficiently deconjugate steroid phase II conjugates. The streamlined sample preparation and high-throughput solid phase extraction in a 96-well plate significantly accelerate sample processing for complex matrices and alarge number of samples. We were able to achieve the necessary sensitivity for accurately measuring the target analytes, particularly estrogens, in small sample sizes of 5–20 mg of liver tissue and 100 μL of plasma. Through the analysis of liver and plasma samples from adult and neonatal rats, including both sexes, our study showed a novel set of steroid hormone reference intervals. This study provides a reliable diagnostic tool for the quantification of steroids in rat models and gives insight in liver and plasma-related steroid hormone dynamics at early developmental stages. In addition, the method covers several pathway intermediates and extend the list of steroid hormones to be investigated.

1. Introduction

Steroid hormones are essential in coordinating and regulating various physiological functions controlled by the endocrine system, including growth, development, sexual differentiation, reproduction, and inflammation [1]. Steroid hormones are determined to provide information on physiological processes, study endocrine disorders, and assess the effects of experimental treatments or interventions on hormone levels [2–4]. Rats have long been the primary model organism in biomedical research and toxicology studies [5–7], making it crucial to develop sensitive methods for characterizing and detecting endogenous steroid hormones in the biofluids and tissues of rats, as this can help identify sensitive biomarkers of adverse effects. The choice of specific subsets of steroid hormones to be measured at a particular developmental stage, sex, and in specific biofluids or tissues relies on the research goals and the particular disease model under investigation. Adult rats, particularly male rats, are commonly favored for studying steroid hormones because of their stable hormonal environment, well-developed endocrine systems, and practical benefits such as larger size, making blood sampling and hormone yield easier to obtain. Additionally, the preference for male rats helps minimize the influence of hormonal fluctuations that are present in female rats due to their estrous cycle [8–10]. Blood, plasma, serum, and urine are the most commonly employed biofluids due to their wide availability, ease of collection, and comprehensive representation of systemic hormone levels. Although utilizing adult male rats simplifies experimental design and result interpretation, it is important to acknowledge the scarcity of steroid hormone reference data in the existing literature for both sexes and at early developmental stages. The liver serves as a central hub for

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Abbreviations

17aE2	17α-estradiol
17bE2	17β-estradiol
17bE2 13C3	17β-estradiol 13C3 (2,3,4–13C3)
17OH-DHP	5α-pregnane 17-hydroxypregnane-3,20-dione
17OH-preg	17α-hydroxypregnenolone
A4	androst-4-ene-3,17-dione (androstenedione)
A4 13C3	androst-4-ene-3,17-dione 13C3 (2,3,4–13C3)
ACN	acetonitrile
AN	5α-androstan-3α-ol-17-one (androsterone)
AN D4	5α-androstan-3α-ol-17-one D4 (2,2,4,4-D4)
And-ediol	5α-androstene-3β,17β-diol (androstenediol)
And-ediol D3	5α-andosten-3β, 17β-diol D3 (16,16,17-D3)
BGS mix	Recombinant β-glucuronidase/sulfatase mix
Cort	corticosterone
Cort D4	corticosterone D4 (9,11,12,12-D4)
DHEA	dehydroepiandrosterone
DHEA D6	dehydroepiandrosterone D6 (2,2,3,4,4,6-D6)
DHT	dihydrotestosterone
DHT D3	dihydrotestosterone D3 (16,16,17-D3)
DOC	11-deoxycorticosterone
EC βGlu	*Escherichia coli* β-glucuronidase
EDCs	endocrine disrupting chemicals
E	cortisone
E D8	cortisone D8 (2,2,4,6,6,9,12,12-D8)
E1	estrone
E1 13C	estrone 13C3 (2,3,4–13C3)
E3	estriol
E3 13C	estriol 13C3 (2,3,4–13C3)
EDC	Internal Standard
Etio	etiocholanone
Etio D5	etiocholanone D5 (2,2,3,4,4-D5)
F	cortisol
F D4	cortisol D4 (9,11,12, 12-D4)
HP ArylS/βGlu	*Helix pomatia* β-glucuronidase/arylsulfatase
IS	Internal Standard
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MeOH	methanol
NS	Native Standard
Preg	pregnenolone
Preg 13C2	pregnenolone 13C2-2D2 (20,21-13C2, 16,16-D2)
Prog	progesterone
prog 13C3	progesterone 13C3 (2,3,4–13C3)
QC	quality control
S	11-deoxycortisol
S D5	11-deoxycortisol D5 (2,2,4,6,6-D5)

the synthesis, metabolism (active/inactive forms), transport and clearance of steroid hormones. The liver produces ~70% of the total daily cholesterol and steroidogenic tissues utilize cholesterol as substrate for the biosynthesis of pregnenolone which is the precursor of all steroid hormones. It also detoxifies substances that can disrupt hormone balance and produces hormone-binding proteins [11–14]. Additionally, in the liver, glucocorticoids have a crucial role in controlling the brain's energy supply through the regulation of gluconeogenesis, glycogenolysis, and the mobilization of fatty acids [15–17]. It is worth noting that the current literature lacks a thorough investigation of steroid hormone levels in rat liver, with no reported data available at early developmental stages [18,19].

When analyzing steroid hormones as disease biomarkers, researchers may focus on either the total fraction or the free fraction, depending on their research goals and the biological significance of each fraction. Analyzing the total fraction allows for the detection of conjugated and bound to protein forms, leading to increased detected concentrations and a more accurate representation of hormonal status [20,21]. Enzymatic deconjugation is often used in sample preparation to remove β-glucuronides and sulfate ester groups, allowing for the quantification of the total fraction. The choice of enzyme for deconjugation is important, and typically, *Escherichia coli* extract or *Helix pomatia* juice are used, but limitations in yield and undesired byproduct formation have been observed, particularly with *H. pomatia* [22–31]. In this study, we conducted a comparative analysis of β-glucuronidase from *E. coli*, β-glucuronidase/arylsulfatase from *H. pomatia*, and a dual recombinant β-glucuronidase/sulfatase mix (BGS mix). Our findings emphasize the reliability of the BGS mix as a viable alternative enzyme. In method development and validation studies, charcoal-activated carbon or solid-phase extraction techniques are often used to obtain a matrix free of steroid hormones [32–35]. Regulatory guidelines recommend using a blank matrix for validation purposes [36,37]. However, concerns arise about the selective removal of steroid hormones during stripping, particularly in avoiding coexisting molecules with similar polarity. These molecules, such as sterols, cholesterol esters, phospholipids, and fatty acids (e.g.), can introduce interferences during steroid hormone analysis [38,39]. Optimizing methods using steroid-free matrices, may lead to underestimation of analytical performance measures such as matrix effect and limits of detection and quantification. To address this issue, our study used unstripped matrices, allowing for a thorough assessment of all potential sources of interferences.

Analyzing steroid hormones in biofluids and tissues presents several analytical challenges due to their low concentration, complex matrix effects, and structural similarities among different steroid molecules [40–43]. While many analytical methods primarily focus on well-known steroid hormones, efforts are being made to expand the repertoire of analyzed steroid hormones to better understand their roles in various physiological processes [44]. To achieve this, gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS) have been the techniques of choice for steroid hormone profiling and quantification [45]. The objective of this study was to optimize and validate a streamlined enzymatic, solid-phase extraction, and LC-MS/MS method for quantifying a panel of 23 steroid hormones in the liver and plasma of adult and neonatal rats. An optimized enzymatic hydrolysis protocol using the recombinant β-glucuronidase/sulfatase mix (BGS mix) is proposed for safe and effective hydrolysis of steroid phase II conjugates. This study introduces a high-throughput workflow in a 96-well plate format, significantly expediting the preparation of complex matrices and large sample sizes. The method's applicability was demonstrated through analysis of liver and plasma samples from adult and neonatal rats of both sexes, and a novel set of reference intervals for steroid hormones is reported. The ultimate goal was to establish a reliable method that can be used as a diagnostic tool for advancing our understanding of steroid hormone dynamics in rat models.

2. Materials and methods

2.1. Chemicals and reagents

Steroid certified reference materials, their labelled internal standards (IS), chemicals, reagents and test materials were purchased from the

following providers: dihydrotestosterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, pregnenolone, testosterone, androst-4-ene-3,17-dione, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, cortisol, progesterone, cortisone, etiocholanone, dehydroepiandrosterone, 5α-androstan-3α-ol-17- one, estrone, estriol, 17β-estradiol, 17α-estradiol, estrone ^{13}C3, 17β-estradiol ^{13}C3, estriol ^{13}C3, 5α-androstan-3,17-dione ^{13}C3, dihydrotestosterone D3, cortisol D4, corticosteroneD4, 11-deoxycortisol D5, etiocholanone D5, dansyl chloride (DNSCL), ammonium fluoride (NH4F), sodium acetate (CH_3COONa), β-Glucuronidase from *E. Coli* (EC bGlu) and β-Glucuronidase/Arylsulfatase from *H. pomatia* (HP bGlu + Aryls) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands); 5α-androstene-3β,17β-diol was purchased from Steraloids (Newport, RI, U.S.), 5α-pregnane 17α-ol-3,20-dione was purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.); 5α-androstan-3,17-dione, 17α-hydroxyprogesterone, cortisone D8, 5α-androstan-3α-ol-17- one D4, 17α-hydroxyprogesterone ^{13}C3, pregnenolone ^{13}C2-2D2 were purchased from Eurisotop (Saint-Aubin, France); testosterone ^{13}C3, dehydroepiandrosterone D6, 5α-androstan-3,17-dione ^{13}C3 and α-andosten-3β, 17β-diol D3 from IsoSciences (Ambler, PA, U.S.). Acetic acid (CH_3COOH), formic acid (HCOOH/FA), HPLC-grade methanol (MeOH) and acetonitrile (ACN) were purchased from Biosolve BV (Valkenswaard, the Netherlands). Purified water was produced using Milli-Q® grade apparatus (Millipore, Bedford, MA, U.S.). The Recombinant β-glucuronidase/sulfatase mix (BGS mix) and the buffer solution in combination (Instant buffer II) were provided by Kura Biotech (Puerto Varas, Chile). Female adult heparinized plasma was acquired from Charles River Laboratories (Wilmington, MA, U.S.).

2.2. Working solutions, calibrators, and quality control samples

Stock solutions for each 23 native steroid standards (NS) and 18 internal standards (IS) were prepared in different solvents and concentrations as reported in Table S1. Multi-analyte intermediate and working solutions were prepared by diluting each individual stocks in MeOH or water. To prepare the calibration curves, a stepwise dilution approach was employed for both the underivatized steroid hormones analysis (12 points) and the dansylated derivatives (6 points). The working solutions were gradually diluted in a combination of water and methanol (in a 1:1 ratio) to obtain the respective calibration curves (concentrations details in Table S2). A solution containing all IS (IS mix) was prepared in MeOH by spiking different volumes of each IS intermediate solution at appropriate concentration per IS (concentration details in Table S3). Since we did not use any blank (dialyzed) matrix, in-house quality controls (QCs) at three test levels, high, middle, and low, were prepared by spiking test plasma and liver samples at specific concentration per steroid. In accordance with International Council for Harmonisation (ICH) and the Food and Drug Administration (FDA) guidelines for endogenous compounds analysis, we first determined the endogenous concentrations of the analytes in plasma and liver test material and then evaluated the concentration spiking ranges for the high, middle, and low QCs [36,37].

2.3. Test material and study samples

To optimize and validate the method, the test material consisted of heparinized pooled plasma from adult female Long Evans rats (11 weeks old) and bovine liver. The plasma was purchased from Charles River Laboratories, while the liver was sourced from a local butcher in Amsterdam. These samples were also utilized for quality control (QC) purposes. Within the context of the ENDpoiNTs and FREIA projects [46,47], we analyzed 25 study samples obtained from Long Evans and Sprague Dawley rats provided by project partners. These samples were employed to evaluate the suitability and effectiveness of the method under investigation. Specifically, the plasma samples were collected from male and female adult rats (9–10 weeks old) and female post-natal day-14 (PND14) pups. As for the liver samples, they were taken from PND6 pups, both male and female. In both cases, 100 μL of plasma and 5–20 mg of bovine or rat liver were used for sample intake. The selection of gender and developmental time points in the study samples was aligned with the research questions addressed in the aforementioned projects. All the experiments and tissue collections were conducted with the approval of the ethical committees of the Complutense University of Madrid and the Danish Animal Experiments Inspectorate [48,49].

2.4. Liver and plasma sample preparation and extraction

The sample preparation workflow for steroid analysis in liver and plasma consists of five steps: a) sample pretreatment (addition of internal standards, water, instant buffer, and homogenization (liver only), b) enzymatic deconjugation, c) steroid pre-extraction/protein precipitation (liver only), d) solid phase extraction (SPE), and e) derivatization of estrogens after 1st LC-MS/MS run. Therefore, these steps were optimized, and the method was validated.

2.4.1. Experimental design enzymatic deconjugation reaction

In this study we compared two of the most used enzyme preparations for the hydrolysis of the steroid phase II conjugates, the arylsulfatase/β-glucuronidase extract from *H. pomatia* (HP ArylS/bGluc) and the β-glucuronidase from *E. coli* (EC bGlu) [24,45,46], to an ultra-pure recombinant β-glucuronidase/sulfatase aqueous mix (BGS mix). The experimental conditions for each enzyme preparation are provided in Table 1. All experiments were performed in triplicate (n = 3) using unspiked plasma (100 μL of pooled adult female rats) and liver samples (5–20 mg of bovine liver). Given that the bGlu activity in *H. pomatia* is relatively low compared to EC bGlu [45], and EC bGlu alone can only hydrolyze steroid glucuronides, we explored the possibility of using EC bGlu and HP ArylS enzymes in combination. According to the manufacturer's instructions, simultaneous use of EC bGlu and HP ArylS is feasible if the reaction is conducted at pH 6.2 (optimal pH range for EC bGlu: 6.0–6.5, optimal pH for HP ArylS: 6.2). However, we needed to assess the optimal incubation temperature for EC bGlu, which can range from 37 °C to 55 °C, and then determined the optimal enzyme amounts for both preparations (Exp. 1 & 2). Regarding the BGS mix, the provider provided guidelines for the optimum pH, incubation temperature, and time for urine samples. However, we further optimized the amount of the BGS mix and the incubation time since these factors may vary depending on the substrate being analyzed. Once the final settings for EC bGlu (Exp. 1), HP ArylS (Exp. 2), the combination of the two (Exp. 3), and the BGS mix (Exp. 4) were determined, we compared the levels of

Table 1
Schematic overview of the experiments performed to optimize the enzymatic deconjugation reaction [a]

Experiment	Treatment	pH	Temperature (°C)	Incubation Time (h)	Amount of Enzymes (μL)
1	No	/	/	/	/
	EC bGluc	6.2	37	1, 3, 22	5, 15
	EC bGluc	6.2	52	1, 3, 22	5, 15
2	No				
	HP ArylS	6.2	37	0, 1, 2, 3, 4, 22	5, 15, 30
3	No	/	/	/	/
	[EC bGluc + HP ArylS]	6.2	37	22	15 + 30
	No	/	/	/	/
4	BGS mix plasma	6.9	52	0.5, 1	100, 200
	BGS mix liver	6.9	*52*	0.5, 1	25, 50, 100

[a] Every experiment was conducted using both untreated and treated matrices under specific enzymatic conditions. For experiments 1, 2, and 3, the same conditions were tested for both plasma and liver samples (plasma 100 μL, liver 5-20 mg, n = 3 per condition).

steroid hormones detected in unspiked plasma and liver samples with and without the specific enzymatic treatments. Statistical analysis was performed using repeated measures ANOVA, followed by Turkey's post hoc analysis.

2.4.2. Optimization pre-extraction and protein precipitation

In order to determine whether an extraction or precipitation step with an organic solvent would improve the detection of steroid hormones in addition to a solid phase extraction (SPE) we conducted a comparison study with different solvents. Specifically, we tested the addition of none, 1:1, and 1:2 (sample intake/organic solvent) of MeOH or ACN to both plasma and liver pretreated solutions or homogenates. When the organic solvent was added at a 1:2 ratio, the samples were allowed to partition in an ice bath for 15 min to facilitate the precipitation process. Subsequently, the samples underwent centrifugation at 17949 rcf and 20 °C for 10 min. The resulting supernatants were then diluted with water containing 2% formic acid (FA) to reduce the amount of organic solvent and promote the pairing of steroid hormones with the SPE sorbent.

2.4.3. Optimized steroid analysis method

The final method for liver and plasma pretreatment and steroid hormones extraction is schematically given in Fig. 1.

3.4.3.1. Sample pretreatment

3.4.3.1.1. Liver. Liver samples (5–20 mg) were added to 1.5 mL Eppendorf tubes, prefilled with ceramic beads. Steroid hormones internal standard mix (50 μL of IS mix prepared in >90% water) and 100 μL of instant buffer were added, and the samples homogenized with a Precellys bead-beating mill (Bertin Instruments, Montigny-le-Bretonneux, France) for three cycles of 10 s, 6500 rpm, with 15 s breaks in between. Samples were placed in an ultrasonic bath for 10 min, before enzymatic reaction.

3.4.3.1.2. Plasma. The high-throughput plasma workflow consisted in the direct transferring of 100 μL of plasma to a 1.2 mL 96 deep well collecting plate (Agilent Technologies, Santa Clara, CA, U.S.), followed by the addition of 50 μL of IS mix, 250 μL of water, and 200 μL of the instant buffer. No sample pretreatment was therefore applied before the enzymatic reaction.

3.4.3.2. Enzymatic deconjugation. Deconjugation of steroid hormones was performed by adding 50 and 100 μL of BGS mix to the liver and plasma pre-treated samples, respectively. The samples were gently mixed by hand before being placed in the oven at 52 °C for 30 min for plasma and 60 min for liver.

3.4.3.3. Pre-extraction. The liver post-enzymes solutions were added with 250 μL of ACN, ultrasonicated in a water bath, and further centrifuged for 10 min at 17949 rcf and 20 °C. The supernatant was diluted with water at 2% FA to reduce the MeOH content to <10%. Water (400 μL) with 2% FA was added to the plasma post-enzyme solutions to quench the enzymatic activity and facilitate the steroid hormones pairing to the SPE sorbent. Therefore, no sample pretreatment with organic solvents was applied after the enzymatic reaction to the plasma workflow.

3.4.3.4. Solid phase extraction. A 96 SPE well plate (VersaPlate, Agilent Technologies, Santa Clara, CA, U.S.) equipped with Bond Elut Plexa 30 mg (1.8 mL) sorbent size cartridges (Agilent Technologies, Santa Clara, CA, U.S.), a vacuum chamber for 96 well plates, and a vacuum pump for SPE were used. The cartridges were conditioned with 1 mL of MeOH 0.5% FA, followed by 1 mL water at 0.5% FA. The liver or plasma pretreated solutions were loaded to the SPE cartridges, prewashed with 0.5 mL of water, and washed with 1 mL of water at 30% MeOH. Vacuum was applied for 10 min to dry the cartridges. The samples were then eluted into a clean polypropylene 96-deep well-collecting plate (Agilent Technologies, Santa Clara, CA, U.S.) with 700 μL for plasma samples and 1 mL for the liver of 100% MeOH; 10 s of vacuum were applied so that all extracts were collected. The eluates were evaporated to dryness in a refrigerated CentriVap vacuum concentrator (Labconco, Kansas City, MO, U.S.) at 40 °C. The dry residues were then reconstituted in 50 μL MeOH/water (1:1) mix and mechanical shaken for 20 min. The plate was then sealed and centrifuged for 2 min (17949 rcf) to let the solution settle down before being placed into the LC-MS/MS autosampler for underivatized steroid hormones quantification.

2.5. LC-MS/MS

2.5.1. Underivatized steroid hormones

The steroid fragmentation patterns were optimized on a triple quadrupole mass spectrometer (SCIEX Triple Quad 6500+ System), equipped with an electrospray ionization source (ESI), operating in positive and negative mode (SCIEX, Framingham, MA, U.S.). Individual steroid hormones were directly injected, and the fragments tested on a ramp of collision energies until the candidate precursor ions (Q1) were identified, and two product ions (Q3) selected based on the optimized compound and source specific parameters (Table S4). The optimized instrument parameters were: curtain gas = 35 psi, temperature = 600 °C, ion source gas 1 = 70 psi, ion source gas 2 = 50 psi, ion spray voltage = 5500 & −4500 V. Separation of steroid hormones was performed with a Kinetex® C18 LC Column (2.6 μm, 100 × 2.1 mm) from Phenomenex

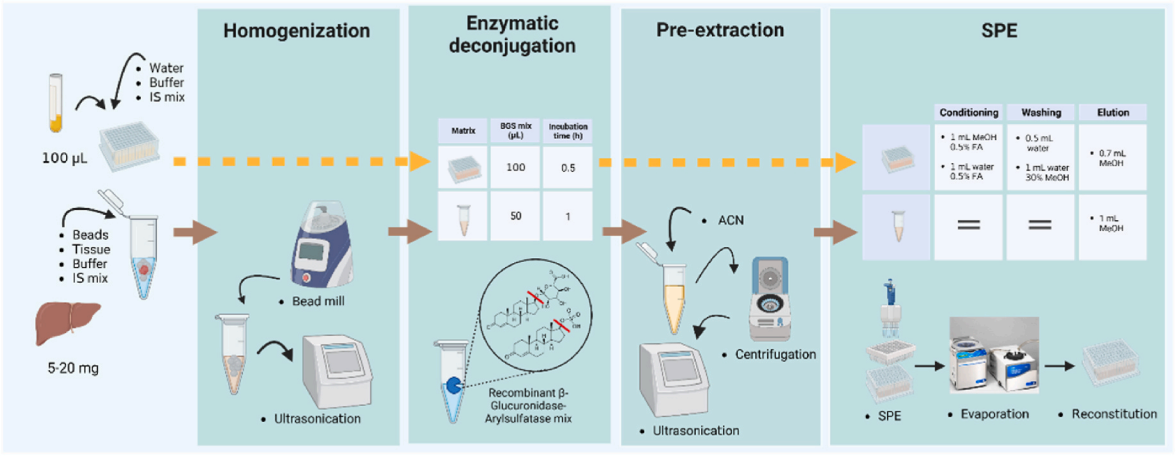

Fig. 1. Schematic representation of the optimized samples pretreatment and steroid extraction in liver (brown arrow path) and plasma (yellow arrow path). Dashed arrows indicate steps that are unnecessary for steroid hormone extraction in plasma.

(Torrance, CA, U.S.), heated at 40 °C on an ExionLC system (SCIEX, Framingham, MA, U.S.) equipped with a binary pump and autosampler. Sample injection volume was 10 μL and the flow rate was 0.6 mL/min. Total LC analysis time was 18.5 min with a solvent gradient of water (A) with 0.2 mM% NH_4F and methanol absolute (B). The elution gradient was as follows: 0–1 min, 20%–50% B; 2–6 min, 50%–60% B, 6–11.9 min, 60%–100% B; 11.9–15.0 min, 100% B; and 15–18 min, 20% B. The analytes were monitored by multiple reaction monitoring (MRM) operating in both polarities; the optimized fragmentation patterns, source parameters and retention times, for both underivatized and dansylated estrogens are listed in Table S4.

2.5.2. Dansylated steroid hormones

In this study, a derivatization reaction with dansyl chloride was carried out to improve the detectability of E1, E3, 17bE2, 17aE2 in the triple quadrupole [50,51]. For the reaction we adapted a protocol reported elsewhere with some minor modifications, which make use of a dansyl solution at 1 mg/mL in acetone, and a carbonate/bicarbonate buffer (pH 10.5) [52]. After the first LC run for underivatized steroid hormones analysis, the samples, and a 6-points calibration curve, obtained by mixing 50 μL of estrogens IS mix with 50 μL of estrogens calibration solutions (150-5 pg/mL), were evaporated to dryness in a refrigerated CentriVap vacuum concentrator (Labconco, Kansas City, MO, U.S.) at 40 °C. Next, 35 μL of dansyl solution and 35 μL of carbonate/bicarbonate buffer (pH 10.5) were added to all residues (samples and calibrators) and the collecting plate was mechanically shaken for 10 min. The plate was then incubated for 4 min at 60 °C before being sealed and placed in the LC-MS/MS autosampler. For dansylated estrogens the same LC method as for the underivatized steroid hormones was adopted, except some minor modifications in the elution gradient (Table S5) needed for a better separation of the estradiol isomers. The dansylated analytes were monitored by positive multiple reaction monitoring (MRM); the optimized fragmentation patterns, source parameters and retention times, are listed in Table S4.

2.6. Method validation

The method validation followed the guidelines provided by the International Council for Harmonisation (ICH) and the Food and Drug Administration (FDA) for bioanalytical method validation [38,39]. Several parameters, including accuracy, precision, matrix effect, calibration model, selectivity, and carryover, were assessed. To prepare the high, middle, and low-quality control samples (QCs), the spiking levels were determined based on the endogenous steroid levels found in the test plasma and liver samples. For steroid hormones that were not detected in the test material, the baseline signal was used for evaluation of the spiking concentrations. Inter-day accuracy and precision were evaluated by analyzing eight replicates of the low and middle QCs in three separate runs. Intra-day accuracy and precision were assessed by repeating the same experiment on a different day and combining the data from all runs conducted on both days. The high QC was only tested for intra-day precision. Extraction recoveries were calculated by comparing the peak areas of the liver and plasma test materials, spiked with target analyte mixes at the three QC levels, before and after extraction. Matrix effects (ME), which indicate ionization suppression or enhancement, were investigated by comparing the peak area of test plasma and liver samples spiked after extraction with that of the corresponding methanolic standard solution containing all target analytes. By defining the peak areas obtained from neat solution standards as A, the peak areas from matrices spiked after extraction as B, and the peak areas for matrices spiked before extraction as C, the ME (Matrix Effect) and RE (Recovery Efficiency) values were determined using the following calculations:

$$\mathrm{ME}\ (\%) = \mathrm{B/A} \times 100$$

$$\mathrm{RE}\ (\%) = \mathrm{C/B} \times 100$$

To assess selectivity, the test samples, both unspiked and spiked, were subjected to extraction and analysis, and the fragment ion ratios were subsequently compared. Isotope-dilution techniques were employed to correct for variations in sample preparation and instrumental response. The peak areas of the analytes were normalized to their isotopically labelled internal standards for quantification. Calibration solutions were prepared for underivatized steroid hormones (12 points) and dansylated derivatives (6 points, covering a narrower concentration range expected to be in the low pg/mL range). These solutions were prepared in a mixture of purified water and methanol (1:1), measured in triplicate, and subjected to least-squares regression analysis to determine the best-fitting calibration model, including the order (linear or quadratic) and weighting (none, 1/x). The goodness of the calibration model was computed in GraphPad Prism and confirmed by the analysis of variance lack of fit. The limit of detection (LOD) and quantification (LOQ) were determined based on signal-to-noise ratios (S/N) of $\geq$3 and $\geq$10, respectively, using either endogenous or spiked concentrations. For endogenous steroid hormones detected at very low concentrations or not detected at all, the LODs were based on the steroid baseline signal or spiking experiments at the approximate LOD concentration. For steroid hormones detected at high ng/mL levels in the test material, LODs were based on the lowest calibrators. Low QC spiking experiments were conducted to evaluate the accuracy at low levels for all analytes in the liver and plasma, except for those endogenous steroid hormones detected at high concentrations in plasma (corticosterone and progesterone). Carryover was determined by injecting a solvent blank multiple times after the highest calibrator and comparing the analyte peak areas (if present) in the solvent blank with the calibrator.

2.7. Statistics and data analysis

LC–MS/MS raw data acquisition and processing were conducted using AB SciexOS Analyst version 1.6.2 (SCIEX, Framingham, MA, U.S.). Statistical analysis and data visualization were performed using GraphPad Prism (Version 9). The absolute steroid hormones amount (pg/extract) were normalized by the sample intake and the levels not detected or below the method LOD were reported as $<$ LOD. Steroid hormone levels were only reported if measured at $\geq$ LOQ, and at least in 3 biological replicates. Group concentrations were transformed (when needed) for statistic evaluation and are shown as mean (n $\geq$ 3) $\pm$ SD, unless specified otherwise. To account for differences in all the optimization experiments and for drawing conclusions about the optimal settings and statistical significance, repeated-measures ANOVA with post hoc Turkey's test were used; data were transformed and tested for normality, p values of 0.05 or lower were considered statistically significant.

3. Results and discussion

3.1. Enzymatic deconjugation reaction optimization

Four main factors play an essential role when optimizing an enzymatic reaction: temperature, time of incubation, pH, and amount of enzyme. The amount of enzymes to be used is expressed as units - Fisherman or Roy units which directly reflect the enzymatic activity - and might be biased by the manufacturer, who tests the enzymes only under certain conditions (e.g., limited steroid hormones and matrix types.). The concentrations of steroid hormones in biological samples are matrix-dependent, thus it is difficult to standardize the reaction parameters or pretend to use the same settings reported in the literature or in the specification sheets without additional evaluation. In this study two of the most used enzyme preparations for the hydrolysis of the steroid phase II conjugates, HP ArylS/bGluc and EC bGlu, were

compared to an ultra-pure recombinant β-glucuronidase/sulfatase aqueous mix (BGS mix). All the enzymatic reactions were optimized (Table 1) in unspiked test plasma and bovine liver, and the detected steroid hormone concentrations (mean ± SD, n = 3) were compared without, with, and within each enzyme treatment for the best yield. Repeated-measures ANOVA, with post hoc Turkey's test, was used to assess statistically significant changes. After testing experimental conditions 1 (Exp. 1) we observed that EC bGluc optimal settings were dependent only by the enzyme amount, which was set at 15 μL in both plasma and liver (data not shown). The tested settings for HP ArylS (Exp. 2) gave unsatisfying or unclear results for some steroid hormones. Specifically, the detected levels of pregnenolone in plasma and DHEA in the liver were statistically significantly lower (17 and 31% respectively) compared to not hydrolyzed samples, when the incubation time and the enzyme amount were increased at 22 h and 30 μL of HP ArylS (Table S3). The levels of 17α-hydroxyprogesterone, androstenedione, and 11-deoxycorticosterone in plasma, and pregnenolone, progesterone, testosterone, corticosterone, androstenedione, 11-deoxycorticosterone, cortisol and cortisone in the liver, were significantly increased at 22 h incubation time and 30 μL HP ArylS (Table S6).

To date, a few studies report the presence of secondary enzymes in the crude extract of *H. pomatia* (e.g., cholesterol oxidase, peptidase),

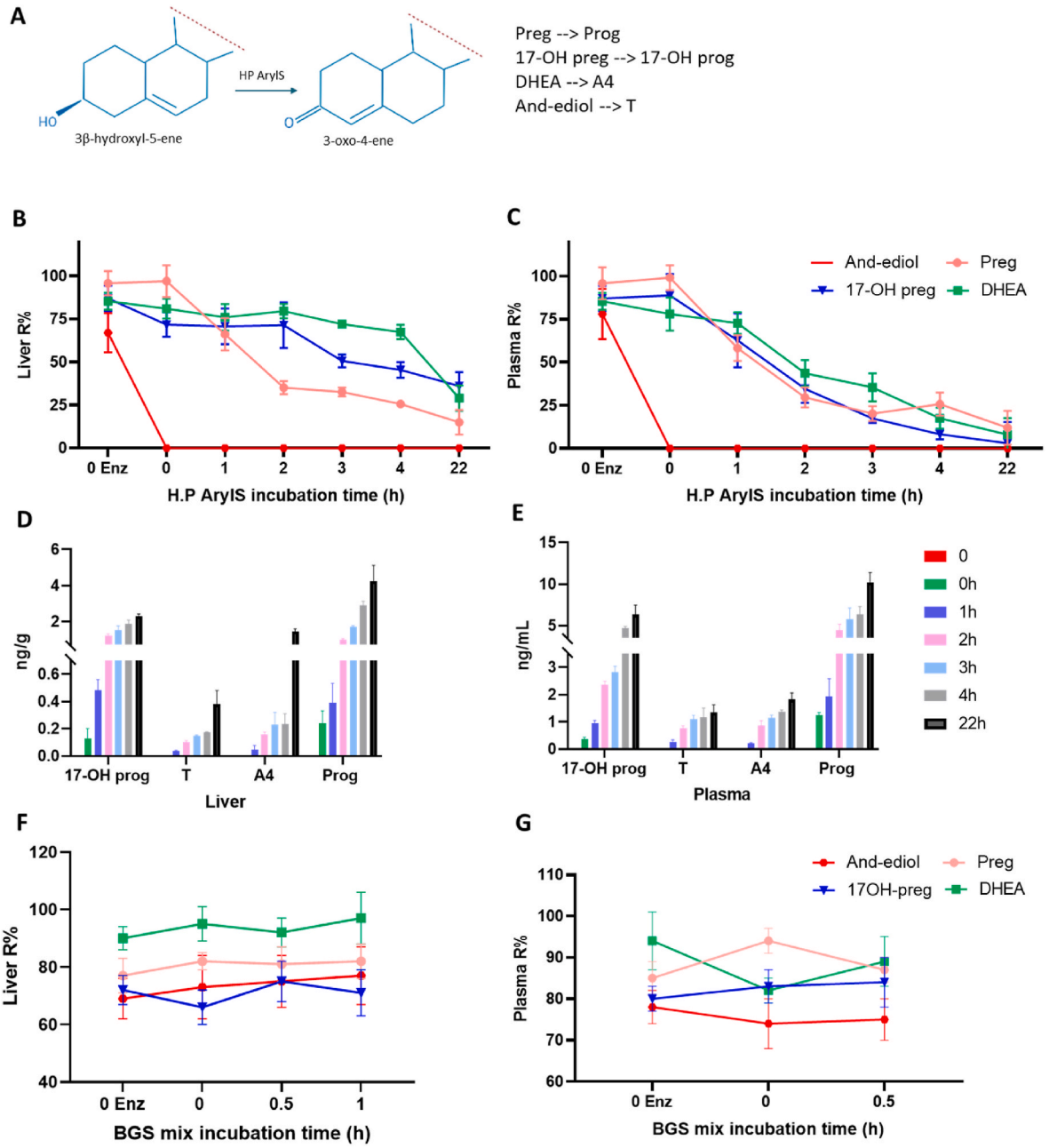

Fig. 2. Enzymatic deconjugation reaction optimization (A) Schematic representation of 3β-hydroxy-5-ene conversion into 3-oxo-4-ene steroid hormones by secondary cholesterol oxydase activity in *Helix pomatia*. (B) and (C) HP ArylS stability test results: decrease of recoveries of pregnenolone (Preg), 17α-hydroxypregnenolone (17-OH preg), DHEA and androstenediol (And-ediol) due to the HP conversion reaction in liver (B) and plasma (C) at different incubation time points. And-ediol (red line) was not detected due to matrix interferences. (D) and (E): Increase of 17α-hydroxyprogesterone (17-OH prog), androstenedione (A4), testosterone (T), progesterone (prog)) levels at increasing incubation time with HP ArylS in liver (D) and plasma (E). (F) and (G) BGS mix stability test results: no decrease of recoveries of pregnenolone (preg), 17α-hydroxypregnenolone (17-OH preg), DHEA and androstenediol (and-ediol) was observed at the tested conditions for both liver (F) and plasmas (G). Concentrations and R % are reported as mean ± SD (n = 3).

which are responsible for steroid conversion reactions, by-products formation, and a misleading estimation of the steroid hormone concentrations [22,25,29–31]. The outcomes of experiment 2 suggested that 3β-hydroxy-5-ene steroid hormones might have been converted into 3-oxo-4-ene steroid hormones, probably because of the presence of secondary enzymatic activity (cholesterol oxidase) in *H. pomatia* extract. Based on our observations, we hypothesized that the reduction in concentrations of pregnenolone and DHEA following HP ArylS treatment could potentially be attributed to their conversion into progesterone and androstenedione, respectively, as shown in Fig. 2A. From the panel of 23 analytes studied, 17α-hydroxypregnenolone and androstenediol also belong to the 3β-hydroxy-5-ene structure and could be a substrate for the conversion reaction (Fig. 2A). To confirm the conversion reaction, spiking experiments for only these four targeted steroid hormones, both in plasma and liver, were conducted, using 15 μL EC bGluc in combination with 30 μL HP ArylS, at 37 °C and at increasing incubation times. The results showed decreased recoveries of the spiked steroid hormones (Fig. 2B and C) and increasing levels of the converted steroid hormones with increasing incubation time, in both plasma and liver (Fig. 2D and E). In addition, androstenediol and its labelled IS were not detected at all after the HP ArylS treatment possibly due to a conversion reaction or matrix interferences deriving form the enzyme, and pregnenolone IS recovery was also unacceptably low (<20%). Next, we tested the BGS mix from Kura Biotech, an high-purity aqueous solution, designed for simultaneous hydrolysis of β-glucuronides and sulfate-conjugates. The BGS specification sheet indicated the optimum reaction conditions and how to adapt the protocol according to the sample intake (enzymes mix/sample ratio 1:1) but only for urine sample analysis. Therefore, the BGS mix amount and incubation time were optimized for both plasma and liver (Exp. 4, Table 1). Whereas the enzymatic hydrolysis did not significantly change the detected steroid hormone levels at increasing incubation time and amount in plasma, in the liver, 17α-hydroxyprogesterone, testosterone, androstenedione, DHEA, 5α-androsterone and 5α-androstanedione were increased, at 1 h incubation time and 50 μL of BGS mix (Fig. S1 and Table S7). In parallel to the BGS mix enzymatic optimization, we also performed recovery experiments, similar to the HP ArylS experiments (Fig. 2B and C), and no conversion into 3-oxo-4-ene steroid hormones of pregnenolone, 17α-hydroxypregnenolone, DHEA and androstenediol, was observed at the optimized conditions of 100 and 50 μL of BGS mix for plasma and liver, respectively (Fig. 2F and G).

Finally, the optimized protocols for the EC bGluc/HP ArylS and BGS mix (Exp. 3 vs. Exp. 4, see Table S8) were compared in plasma and liver. Both enzyme preparations gave similar yields in plasma for corticosterone, progesterone, androsterone and 17β-estradiol concentrations. In the EC bGluc + HP ArylS hydrolyzed samples, 17α-hydroxyprogesterone and androstenedione concentrations were higher than in the BGS mix samples, whereas pregnenolone and testosterone were higher in the BGS mix samples, and estrone was detected in plasma only after the BGS mix treatment (Fig. 3A). In the liver samples, the detected concentrations of testosterone, cortisol, progesterone, and androstanedione were higher after the EC bGluc/HP ArylS treatment, whereas DHEA was higher after

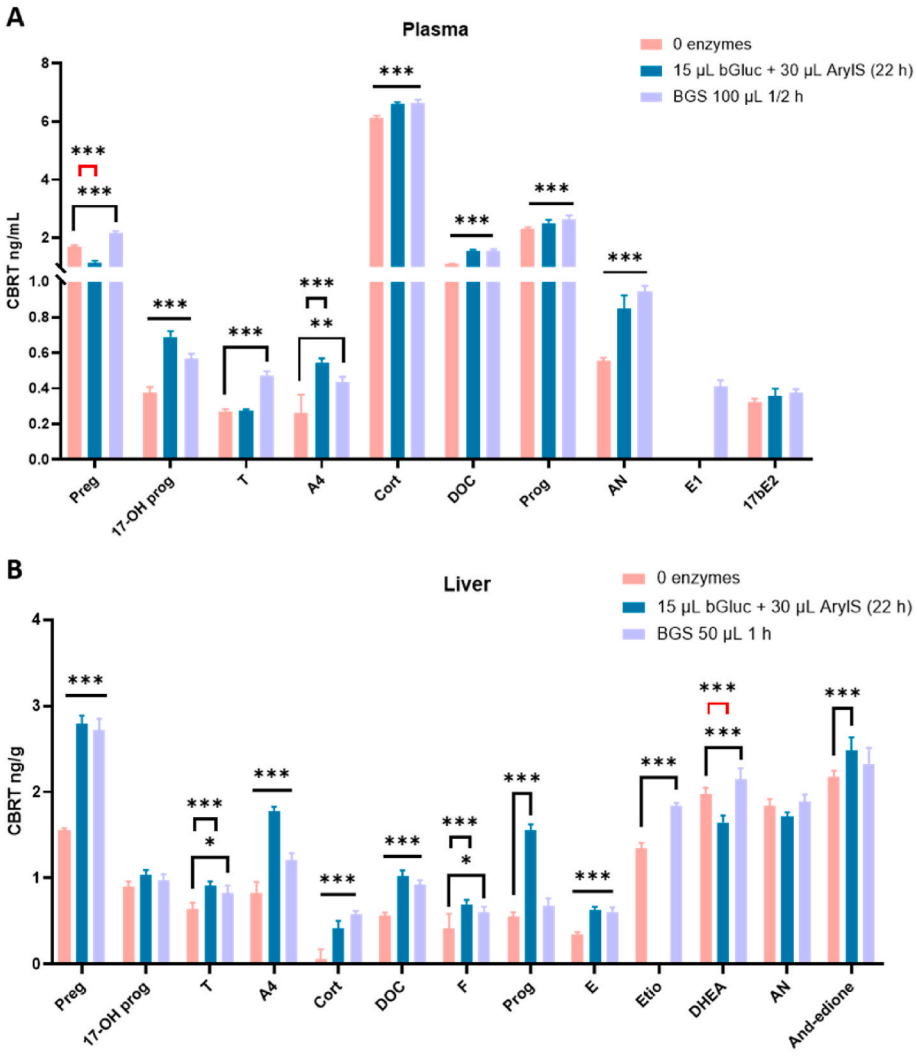

Fig. 3. Cube root transformed concentrations (n = 3, mean ± SD) of the steroid hormones detected after no enzymatic treatment (0 enzymes), EC bGluc 15 μL + HP ArylS 30 μL optimized protocol (Exp. 3), and BGS mix optimized protocol (Exp. 4) in plasma (A) and liver (B). For visualizing statistical significance, a straight line (same level of significance) or a square bracket (specific treatment significance), are used. Black and red square brackets are used for an increase or a decrease in the steroid hormones' concentrations respectively. *p value < 0.03, **p value < 0.002, ***p value < 0.001 (ANOVA details are reported in Table S8).

the BGS mix treatment. Etiocholanone was detected only with no enzymes or the BGS mix treatment (Fig. 3B). Although these findings suggest a higher deconjugation ability of the EC bGluc/HP ArylS treatment for some steroid hormones, we concluded that this was likely a misleading result caused by conversion reactions and byproduct formation. The spiking experiments with the targeted steroid hormones (Fig. 2) indeed confirmed the occurrence of conversion reactions mediated by secondary enzymes, and the HP ArylS inapplicability for steroid hormone analysis in rat plasma and liver at different experimental conditions. Noteworthy, no interferences for the detection of any native or labelled steroid standards were observed after the hydrolysis with the BGS mix. Moreover, the BGS mix is a high-purity product and needed a low incubation time for both plasma (30 min) and liver (1 h). These results indicate that the BGS mix is a valid and reliable alternative for steroid hormones phase II enzymatic deconjugation compared to the crude mixtures of enzymes such as β-glucuronidase/arylsulfatase from *H. pomatia*.

3.2. Steroid hormones pre-extraction and protein precipitation

Steroid hormones are usually isolated from biological matrices by extraction with organic solvents, in which they readily dissolve. Adding organic solvents at a ratio of at least 1:2 (sample intake/organic solvent) promotes the precipitation and removal of proteins, which is substantial in both plasma and liver and represents a source of interference for steroid hormones ionization and affects the detectability in the triple quadrupole [38,39]. However, the literature frequently highlights the recurring challenges of lengthy and laborious sample preparation and clean-up procedures for steroid hormone analysis in complex matrices. Particularly, the protein precipitation step proves to be a laborious and time-consuming process, especially when dealing with a substantial number of samples [18,19,53]. The effect of precipitation of proteins and pre-extraction on steroid hormone recoveries was therefore tested by adding to both spiked plasma and liver pre-treated samples 1:1 and 1:2 of MeOH or ACN (sample intake/organic solvent), and the results were compared to samples where no organic solvent was added. Recoveries of the 23 steroid hormones in QC spiked samples after each treatment are shown in Fig. 4. After testing for statistical significance, the recoveries obtained at each treatment, we observed that the addition of organic solvent did not improve the steroid hormones extraction or protein precipitation processes in plasma. This was probably because the circulating steroid hormones fraction can easily interact with the methanolic environment during the SPE, which efficiently promotes the steroid bound-to proteins breakdown. However, to release steroid hormones that are bounded to the tissue/cell compartments or proteins in the liver, adding ACN to the homogenates (1:1), followed by ultrasonication, is needed. In both plasma and liver, an increase of the organic phase ratio to 1:2 (sample intake/organic solvent) significantly decreased the recoveries of the extracted steroid hormones and did not improve the clean-up (see Table S6).

3.3. Method validation

The method was validated in accordance with ICH and FDA guidelines [38,39]. Before starting the validation experiments, preliminary analysis of liver and plasma test samples were carried out for evaluating

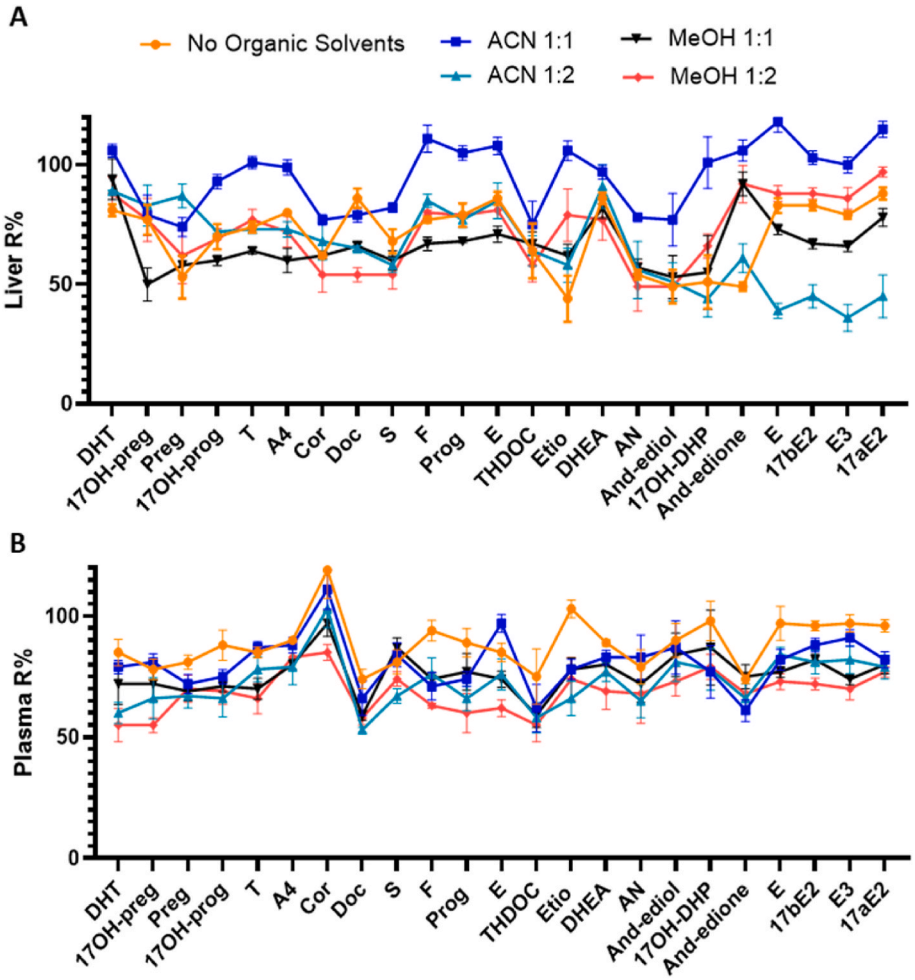

Fig. 4. Steroid hormones recoveries % (mean ± SD, n = 3) after pre-extraction and protein precipitation using no or different amounts of ACN or MeOH (1:1 and 1:2 sample intake/organic amount). In the liver (A), statistically significant higher recoveries (blue line) were observed using a ratio 1:1 sample intake/ACN, compared to no organic solvent treatment; recoveries in plasma (B) showed no improvement after any organic solvent sample pretreatment. ANOVA details can be found in Table S9.

the endogenous concentration and for estimating the spiking levels for LOD assessment when possible. Least-squares regression analysis was performed to test which model would fit at the best the calibration curve, in both underivatized steroid hormones and dansylated derivatives, including the order (linear or quadratic) and weighting (none, 1/x). The goodness of the calibration model was assessed through analysis of variance lack of fit and the calibration model parameters are given in Table 2. A p value above 0.05 indicates the homogeneity of variance of the residuals and no statistically significant deviation from the selected calibration model at the specific range of concentrations. Experimental assessment of LODs involved spiking the test matrices with the targeted analytes at concentrations approximating the LOD and verifying that the signal-to-noise ratio (S/N) was between 3 and 10. For corticosterone and progesterone which were detected at high concentrations in plasma, the LODs were based on the signal of the lowest calibrators. LOD values ranged from 0.01 to 3.0 ng/mL in plasma and 0.01–21 ng/g in liver (Table 2). LOQs were based on the signal-to-noise ratio (S/N) equal or higher than 10. Both accuracy and precision were evaluated on three QC levels, high, middle, and low, which were extracted and measured in 8 replicates and in 3 analytical runs per day (intra-assay) and on two different days (inter-assay) except for the high QC precision that was tested on a single day. The obtained method validation metrics are given in Table 3. For all steroid hormones, the calculated extraction recoveries were between 70% and 113% in plasma and between 61 and 134% in liver at the three concentrations tested. As indicated in the official guidelines for analytical methods validation the goal of recovery experiments is not 100% but either the analytes detection consistency or reproducibility [36,37]. Acceptance criteria for intra and inter-day accuracy and precision (CV < 15%) were indeed satisfactory for all the steroid hormones in both matrices. The ionization suppression/enhancement was measured by comparing the post-extraction spiking experiments and was estimated to be approximately 25% in plasma (except androsterone for which a higher suppression of 32% was observed), and approximately 30% in the liver. The method was validated on intact plasma and liver thus additional sources of interference must be considered for a stronger matrix effect and lower recoveries for some steroid hormones. The carry-over was evaluated by running a solvent blank after multiple injection of the highest calibrators and was not detected for any measured steroid hormones. The optimized sample preparation protocol based on solid-phase extraction (SPE), along with optimized chromatographic separation, and the careful selection of up to two multiple reaction monitoring (MRM) transitions for each target analyte, contributed to the selectivity in the method (Tab S3). The ion ratio between the quantifier (Q1) and qualifier (Q3) ions and the presence of interfering peaks around the expected retention time were evaluated in the test samples as well. For the steroid hormones for which we had no IS, marked with an asterisk (Table 3), we used an analogue IS with a similar elution time window and chemical structure. The robustness of the method was evaluated throughout a two-day validation, involving two different operators, repeating the analyses in 3 different batches. The test samples were re-extracted on each validation day as well as the mobile phases and solutions were freshly prepared.

3.4. Application to study samples

Despite rats being extensively studied as primary model organisms in biomedical research, there remains a necessity for sensitive methodologies to characterize and detect a comprehensive set of endogenous steroid hormones in rats' biofluids and tissues. Due to their hormonal stability and absence of fluctuations associated with female reproductive physiology, adult male rats are commonly favored as a model for hormone studies [8–10]. Additionally, the continuous developmental changes during early stages pose challenges in accurately measuring and analyzing hormone levels, further supporting the preference for adult male rats [54]. Despite the advantages of using adult male rats in experimental design and result interpretation, it is important to recognize the scarcity of steroid hormone reference data in the current literature for both sexes and at early developmental stages. Blood, plasma, and urine are the most studied matrices for steroid hormone analysis due

Table 2
Calibration model parameters and method LODs in test plasma and liver for the panel of 23 steroid hormones [a].

Analytes	Calibration range ng/mL	Polynomial Model	Weight	Goodness of fit			LOD	
				R^2	Sum of Squares	LoF (p value)	Plasma (ng/mL)	Liver (ng/g)
DHT	[0.05–165]	linear	1/x	0.994	0.7	0.5	0.1	0.6
Preg	[0.6–172]	linear	1/x	0.995	0.9	0.5	2.2	5.5
17OH-prog	[0.01–126]	linear	1/x	0.998	0.5	0.2	0.05	0.05
T	[0.01–60]	linear	1/x	0.998	0.3	0.5	0.01	0.06
A4	[0.01–60]	linear	1/x	0.999	0.1	0.09	0.02	0.04
Cort	[0.06–179]	quadratic		0.999	0.7	0.7	0.5	1.4
F	[0.01–165]	linear	1/x	0.998	1.1	0.5	0.06	0.3
Prog	[0.02–110]	linear	1/x	0.997	0.7	0.06	0.3	0.2
E	[0.01–155]	linear	1/x	0.995	1.4	0.4	0.01	0.2
Etio	[0.65–185]	linear	1/x	0.997	0.1	0.08	1.0	2.6
DHEA	[0.50–150]	linear	1/x	0.998	0.6	0.9	0.5	0.4
AN	[0.19–160]	linear	1/x	0.998	0.1	0.1	0.3	2.4
And-ediol	[1.25–178]	quadratic		0.999	0.1	0.4	3.0	21
And-edione	[0.70–185]	linear	1/x	0.999	0.2	0.6	0.7	12
17OH-preg	[1.27–129]	linear		0.999	5.0	0.1	0.6	1.6
DOC	[0.01–60]	linear	1/x	1.000	0.04	0.2	0.3	0.2
S	[0.01–120]	linear	1/x	0.997	4.9	0.2	0.6	0.2
THDOC	[1.24–130]	linear		0.995	0.06	0.7	1.4	4.8
17OH-DHP	[0.21–130]	quadratic		0.999	0.03	0.3	0.2	2.5
E1	[0.11–113]	linear	1/x	0.999	0.01	0.8	0.05	0.1
17bE2	[0.22–180]	linear	1/x	0.999	0.07	0.8	0.06	0.1
17aE2	[0.13–60]	linear		0.998	0.02	0.1	0.05	0.1
E3	[0.06–175]	linear	1/x	0.999	0.7	0.3	0.06	0.1
17aE2-dns	[0.01–0.07]	linear		0.994	3.66E-07	0.9	0.02	0.02
E1-dns	[0.002–0.07]	linear		0.993	1.21E-06	1.0	0.02	0.01
E3-dns	[0.003–0.07]	linear		0.999	1.74E-05	0.8	0.01	0.03
17bE2-dns	[0.003–0.07]	linear		0.993	1.01E-06	0.6	0.01	0.04

[a] A p value > 0.05 indicate the homogeneity of variance of the residuals and no significant deviation from the selected calibration model at the specific range of concentrations (ANOVA-LoF). dns: dansylated.

Table 3
Method validation parameters for plasma and liver at high, middle, and low QCs levels [a].

Analytes		Plasma					Liver				
	QC	Conc. (ng/mL)	R %	Intra-assay CV %	Inter-assay CV %	ME	Conc. (ng/g)	R %	Intra-assay CV %	Inter-assay CV %	ME
DHT	High	19	93.2	6.2		86	21.7	106.3	7.3		102
	Middle	1.8	89.3	5.5	6.3		7.0	93.4	5.0	8.9	
	Low	0.7	77.1	7.3	8.6		1.3	96.8	8.5	9.6	
17OH-preg*	High	48	101.3	5.9		112	24.4	96.2	7.2	9.0	124
	Middle	12	108.2	6.6	9.0		6.8	89.1	7.2	9.0	
	Low	1.4	83.7	11.8	11.4		2.5	89.8	12.3	14	
Preg	High	53	97.4	4		70	57.3	89.3			76
	Middle	22.2	89.3	8	9.5		12.2	77.3	13.3	11.4	
	Low	5	82.3	11.7	13.8		7.0	75.7	12.8	14.2	
17OH-prog	High	10	97.2			102	53.8	115.2	6.8		109
	Middle	0.4	88.4	4.0	11.9		13.0	106.7	7.3	8.9	
	Low	0.2	88.7	13.2	14		0.2	99.2	11.9	13	
T	High	9.7	94.3	5.7			21.0	116.4	9.5		
	Middle	2	91.5	4.2	6.2	100	3.3	109.3	10.3	11.9	119
	Low	0.1	83.2	9.2	11		0.1	96.9	13.0	15.4	
A4	High	8.9	86.8				52.0	122.7	9.2		
	Middle	2	92.7	8.7	11	117	11.0	115.3	8	13.1	123
	Low	0.1	94.1	12	13.5		0.1	98.5	15	18	
Cort	High	84.2	95.3	3.9	10.3	116	48.0	114.3	6		
	Middle	» LOD					7.9	115.8	4.5	6.4	72
	Low	» LOD					4.4	108.6	8.1	11.9	
DOC*	High	55	108.5	6.5		104	19	117.7	7.2		71
	Middle	22.9	113.2	8.2	4.5		4.0	116.3	8.4	11.3	
	Low	4.8	77.6	7.5	7.8		0.5	93.1	10.4	13	
S	High	20	103.3			88	49	123.0	5.7		119
	Middle	6.4	95.7	3.8	8.9		9.7	117.2	7.8	9.3	
	Low	1.3	88.2	10.9	12		1.2	74.4	10.8	12.5	
F	High	10				101	59.1	112.5	3.1		129
	Middle	0.8	93.6	4.9	10.4		10	96.3	5.3	8	
	Low	0.2	89.1	8.4			1.2	82.1	7.8	10.1	
Prog	High	88.9	94.6	4.5	6.0	99	76.1	104.6	2.4		130
	Middle	»LOD					10	93.4	4.4	5.5	
	Low	»LOD					0.7	106.4	12.5	14.7	
E	High	9.5	106.8	3.5		103	72.7	114.4	2.7		122
	Middle	0.3	83.3	9.8	8.1		11.0	102.8	6.3	9.4	
	Low	0.1	90.5	9.4			1.0	106.9	5.0	6.2	
THDOC*	High	19.7	102.3	8.4		101	48.0	118.3	11		
	Middle	4.2	83.6	7.9	9.1		10.8	100.8	7.6	11.9	129
	Low	1.9	63.7	12.1			7.4	107.5	9.4	13.8	
Etio	High	19	89.2	9.5		95	53.9	101.3	7.6		117
	Middle	9	81.1	11.5	12.9		11.0	97.6	5.3	8.5	
	Low	2.6	74.7	11.0	14		7.4	107.1	9.4	10.2	
DHEA	High	20	96.9	7.2		99	69.9	95.1	8.8		96
	Middle	8.8	93.5	5.9	13.8		12	92.4	11.6	12.2	
	Low	1.7	88.5	10.8	14		4.4	104.6	13.6	14.6	
AN	High	50	72.2	3.8		68	57	96.3	6.2		127
	Middle	30.7	73.8	5.5	7.3		19	77.5	9.5	13.6	
	Low	6.9	70.3	10.7	12		3.8	61.2	12.9	15.7	
And-ediol	High	123.9	89.6	11		125	96.1	100.8	8.9		134
	Middle	48	93.1	6.8	12.7		47.1	106.4	9.6	11.4	
	Low	14.2	75.2	8.7	15.2		22	87.2	11.7		
17OH-DHP*	High	10	78.4	9		75	87	109.3	7.2		
	Middle	1.5	96.4	6	12.3		31.8	91.1	5.2	7.6	116
	Low	0.6	81.7	14.3	15.4		6.4	111.7	14.9	16.2	
And-edione	High	22	84.4	4.0		95	63.9	107.6	9.5		137
	Middle	3.3	88.6	3.8	5.2		31.8	62.3	12.4	13.7	
	Low	1.2	67.9	8.2	11		18	65.1	11.3	14.6	
E1	High	5.0	104.4	3.9		97	23	108.5	4.5		112
	Middle	0.5	98.9	8.1	7.6		4.8	90.2	5.1	5.5	
	Low	0.1	86.7	7.6			0.2	93.1	11.1	13.8	
17bE2	High	6.0	110.3	5.2		95	9.2	90.4	3.7		102
	Middle	0.8	87.4	7.4	8.5		1	96.6	6.3	8.2	
	Low	0.1	83.8	11.6	13.5		0.1	94.1	8.3	10.2	
E3	High	4.0	103.4	7.0		99	11	102.8	8.8		108
	Middle	0.6	95.1	10.2	8.3		1	106.3	5.3	7.6	
	Low	0.1	88.7	7.3	9.8		0.1	106.7	7.7	9.4	
17aE2*	High	5.0	110.2	8.3		89	9	110.1	5.2		103
	Middle	0.6	83.1	10.9	10.9		1	103.4	4.6	7.9	
	Low	0.1	80.4	9.5	11		0.1	85.8	11.7	12.5	

[a] The asterisk is used to indicate the compounds for which the labeled internal standards was not available, and an analogue IS was used.

to their easy accessibility, representation of circulating hormones, and relevance in assessing hormone clearance. Although the liver plays a central role in the synthesis, metabolism, transport, and clearance of steroid hormones [11–14], the current literature lacks a thorough investigation of steroid hormone levels in rat liver, with no reported data available at early developmental stages [18,19]. To address this gap, the objective of this study was to optimize and validate an LC-MS/MS method capable of quantifying a panel of 23 steroid hormones in the plasma and liver of adult and postnatal rats, including both genders. The suitability of the method was therefore assessed by examining 25 samples obtained from the ENDpoiNTs and FREIA projects [46,47]. The study samples included plasma from adult male and female rats, as well as plasma from female rats on postnatal day 14 (PND14), and liver tissues from male and female rats on postnatal day 6 (PND6). The selection of sex and developmental stage for the samples aligned with the research objectives of the respective projects. The concentrations of steroid hormones were detected through the optimized method (mean ± SD, n = 5), and the results, grouped according to gender, age, and matrix type are shown in Table 4. As expected, a broad range of steroid hormones was detected, and the suitability of the analytical method was confirmed for almost all the analyte targets. From the panel of 23 steroid hormones, only 5α-androstan-3,17-dione and tetrahydrodeoxycorticosterone, were not detected in any of the samples. In addition, the four estrogens were all detected and confirmed the method sensitivity in the low ng/mL range (Fig. 5). The differences in steroid hormone levels and matrix types reflect the distribution (circulating and organ-specific forms), sexual maturation and age, in addition to the inter/intraindividual variability in the production, circulating levels and metabolic clearance rates at the time of sampling. During the early stages of development in rats, the production and secretion of steroid hormones are relatively low, and the levels gradually increase with sexual maturation [54]. Through the analysis of matrices collected at postnatal day 6 and 14, we successfully captured steroid hormone dynamics during a critical time window of development. The significance of these findings lies in their novel exploration of steroid hormones during early time points for both genders, as well as in unconventional matrices such as the liver, where no data is currently available in the existing literature. However, a limitation of this study is the relatively low number of samples collected from both male and female rats and for both matrices. Consequently, further validation with larger study cohorts is required to enhance the robustness of the obtained results.

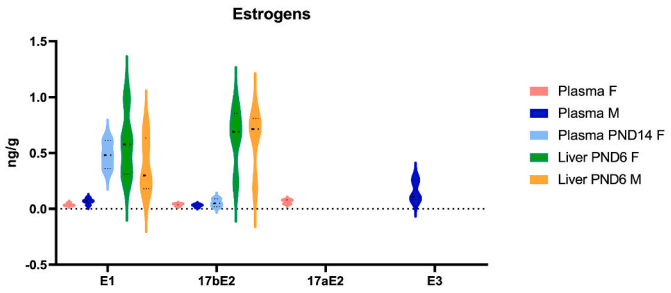

Fig. 5. Violin plots showing the median, interquartile range and distribution of the estrogens concentrations detected in the various matrices (n = 5). The y axis reports the concentration ranges assuming 100 μL of plasma corresponds to 0.1 g of wet tissue.

4. Conclusions

In this study, we optimized and validated an efficient enzymatic, solid-phase extraction, and LC-MS/MS method for accurately measuring a panel of 23 steroid hormones in the liver and plasma samples obtained from adult and neonatal rats. The method encompasses major classes of steroid hormones, including active forms and various pathway intermediates. By implementing a high throughput workflow in a 96-well plate format, we were able to complete a full-plate pretreatment and extraction in approximately 4 h for plasma samples and 6–8 h for liver samples. To ensure reliability and account for matrix complexity, we employed un-stripped bovine liver tissue and rat plasma during the method optimization and validation process. Furthermore, we assessed two commonly used enzymatic preparations for steroid hormone's phase II deconjugation reaction: *Escherichia coli* β-glucuronidase and *Helix pomatia* arylsulfatase extracts. Our findings confirmed the inapplicability of *Helix pomatia* for deconjugating several steroid hormone sulfates [29,30]. As an effective alternative for the enzymatic hydrolysis of both glucuronides and sulfates, we propose the utilization of a recombinant β-glucuronidase/sulfatase mix. This alternative offers the advantages of a high-purity product, thereby preventing steroid

Table 4
Overview of the average concentrations of steroid hormones detected in liver and plasma of adult and postnatal rats (n = 5, per each group).

Analytes	Plasma (adult) Females		Plasma (adult) Males		Plasma PND14 Females		Liver PND6 Females		Liver PND6 Males	
	average (ng/mL)	SD	average (ng/mL)	SD	average (ng/mL)	SD	average (ng/g)	SD	average (ng/g)	SD
DHT	< LOD		0.2	0.02	< LOD		< LOD		< LOD	
T	0.02	0.01	7.6	1.1	< LOD		< LOD		0.1	0.06
Prog	40	9.5	2.3	1.8	2.5	0.9	0.6	0.1	1.7	0.4
17OH-prog	0.09	0.02	0.7	0.1	< LOD		< LOD		< LOD	
A4	0.04	0.01	0.1	0.06	0.03	0.02	0.4	0.5	0.2	0.1
Cor	328	44	245	22.3	11	4.6	5.5	0.9	< LOD	
Preg	7.4	1.7	< LOD		2.5	0.5	< LOD		< LOD	
Etio	4.4	1.0	1.7	0.1	< LOD		< LOD		< LOD	
DHEA	3.6	1.9	< LOD		< LOD		3.8	1.4	< LOD	
F	0.05	0.02	4.4	0.4	< LOD		< LOD		< LOD	
E	< LOD		< LOD		< LOD		1.46	0.3	2.14	1.1
AN	0.8	0.2	0.6	0.2	1	0.4	< LOD		< LOD	
And-ediol	75	18	< LOD		< LOD		< LOD		< LOD	
And-edione	< LOD		< LOD		< LOD		< LOD		< LOD	
17OH-preg	< LOD		4.3	1.2	< LOD		8.4	3.2	14.4	5.8
17OH-DHP	0.4	0.2	< LOD		0.4	0.19	39.1	8.9	98.5	33.0
DOC	10	4.3	8.6	1.6	< LOD		< LOD		< LOD	
S	< LOD		0.6	0.3	< LOD		< LOD		< LOD	
THDOC	< LOD		< LOD		< LOD		< LOD		< LOD	
E1	0.04	0.01	0.07	0.02	0.48	0.1	0.3	0.2	0.4	0.3
17bE2	0.04	0.01	0.03	0.01	0.05	0.04	0.7	0.3	0.6	0.3
17aE2	0.07	0.02	< LOD		< LOD		< LOD		< LOD	
E3	< LOD		0.2	0.1	< LOD		< LOD		< LOD	

conversion reactions and a shorter incubation time. Overall, this optimized and validated LC-MS/MS method provides a reliable diagnostic tool for advancing our understanding of plasma and liver steroid hormone dynamics in rat models; it contributes to expanding the repertoire of analyzed steroid hormones for both sexes and at early stages of development and to investigate previously overlooked matrices, such as the liver.

Credit authors statement

Sara Evangelista: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, Writing – original draft. Paraskevi Vazakidou: Methodology, Investigation. Jacco Koekkoek: Methodology, Supervision. ManuelT. Heinzelmann: Validation, Formal analysis. Walter Lichtensteiger: Resources. Margret Schlumpf: Resources. Jesus A.F. Tresguerres: Resources. Beatriz Linillos-Pradillo: Resources. Majorie B.M. van Duursen: Project administration, Funding acquisition, Writing – review & editing. Marja H. Lamoree: Supervision, Writing – review & editing. Pim E. G. Leonards: Project administration, Funding acquisition, Resources, Supervision, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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