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High-throughput analysis of 19 endogenous androgenic steroids by ultra-performance convergence chromatography tandem mass spectrometry

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- A high throughput UPC^2-MS/MS method for the analysis of 19 steroids is described
- UPC^2-MS/MS offers superior selectivity and increased chromatographic efficiency
- Significant improvements in sensitivity were achieved by UPC^2-MS/MS
- UPC^2-MS/MS is a SFC based technology which is ideal for steroid analysis

Abstract

11-oxygenated steroids such as 11-ketotestosterone and 11-ketodihydrotestosterone have recently been shown to play a putative role in the development and progression of castration resistant prostate cancer. In this study we report on the development of a high throughput ultra-performance convergence chromatography tandem mass spectrometry (UPC^2-MS/MS) method for the analysis of thirteen 11-oxygenated and six canonical C19 steroids isolated from a cell culture matrix. Using an Acquity UPC^2 BEH 2-EP column we found that UPC^2 resulted in superior selectivity, increased chromatographic efficiency and a scattered elution order when compared to conventional reverse phase ultra-performance liquid chromatography (UPLC). Furthermore, there was a significant improvement in sensitivity (5-50 times). The lower limits of quantification ranged between 0.01 to 10 ng mL^{-1}, while the upper limit of quantification was 100 ng mL^{-1} for all steroids. Accuracy, precision, intra-day variation, recovery, matrix effects and process efficiency were all evaluated and found to be within acceptable limits. Taken together we show that the increased power of UPC^2-MS/MS allows the analyst for the first time to complete *in vitro* assays at biologically relevant concentrations and in so doing determine the routes of steroid metabolism which is vital for studies of androgen responsive cancers, such as prostate cancer, and could highlight new mechanisms of disease progression and new targets for cancer therapy.

Keywords: Adrenal androgens; Gas chromatography; Steroids; Supercritical fluid chromatography; Ultra performance convergence chromatography; Ultra performance liquid chromatography

Abbreviations:

3α-adiol, 5α-androstane-3α,17β-diol; 5α-dione, 5α-androstanedione; 11K-3α-adiol, 11keto-5α-androstane-3α,17β-diol; 11K-5α-dione; 11keto-5α-androstanedione; 11KA4, 11-ketoandrostenedione; 11KAST, 11-ketoandrosterone; 11KDHT, 11-ketodihydrotestosterone; 11KepiAST, 11-ketoepiandrosterone; 11KT, 11-ketotestosterone; 11OH-5α-dione, 11β-hydroxy-5α-androstanedione; 11OHA4, 11β-hydroxyandrostenedione; 11OHAST, 11β-hydroxyandrosterone; 11OHDHT, 11β-hydroxydihydrotestosterone; 11OHepiAST, 11β-hydroxyepiandrosterone; 11OHT, 11β-hydroxytestosterone; A4; androstenedione; DHT, 5α-dihydrotestosterone; DRSP, drospirenone; epiAST, epiandrosterone; GES, gestodene; T, testosterone

1 Introduction

Steroids hormones play a vital role in the regulation of a number of physiological processes, which include the regulation of water and electrolyte balance, metabolism, stress response, inflammation and reproductive functions [1]. In addition, endocrine disorders such as congenital adrenal hyperplasia (CAH), Cushing's syndrome, polycystic ovary syndrome (PCOS), primary aldosteronism and hormone dependent cancers, as well as prostate and breast cancer are associated with the dysregulated production of steroid hormones[2–6]. Accurate quantification of steroid hormones is therefore essential for both diagnostic and research purposes.

All steroid hormones are derived from cholesterol and share the basic cyclopentanoperhydrophenanthrene ring structure. The physiological function of individual steroid hormones is determined primarily by modifications to the basic 4-ring steroid structure such as the position of specific double bonds, or the position of hydroxyl or keto functional groups [1]. These subtle structural differences, unique to each steroid hormone, significantly complicate the separation of such structurally similar molecules, which include isobaric species. Although good resolution and sensitivity are achieved by gas chromatography-mass spectrometry (GC-MS), this requires laborious sample preparation, including hydrolysis and derivatisation, and is not applicable to high-throughput assays. While liquid chromatography tandem mass spectrometry (LC-MS/MS) does not achieve the resolution offered by GC-MS, in most cases LC-MS/MS does not require sample derivatisation and ultra-high performance liquid chromatography (UHPLC) instrumentation offer significantly reduced runtimes compared to traditional LC [7,8].

Enhanced chromatographic efficiency and resolution are achieved by supercritical fluid chromatography (SFC) which, due to the unique properties of supercritical fluids (SFs), is able to combine the benefits of both GC and LC. SFs have liquid-like densities and dissolving capacities, but demonstrate high gas-like diffusivity and low viscosities [9–11]. The idea of converging ultra-high pressure gas chromatography with classical liquid chromatography was first proposed by Giddings in 1964 [11]. The convergence of a supercritical mobile phase with liquid organic modifiers maintains the benefits of SFC, with the additional benefits of increased versatility and selectivity [12]. Reproducible analytical output by convergence chromatography is dependent on the ability to control key parameters such as temperature, pressure and density, which, being technically difficult, was only recently achieved, with advances in technology resulting in commercially viable convergence systems [11]. The Waters Ultra-Performance Convergence Chromatography™ (UPC^2) system used in this study makes use of highly miscible supercritical CO_2 which can be modified by the addition of organic solvents with relative polarities ranging from that of for example hexane (0.009) to methanol (0.762) [13]. Furthermore, the compatibility of the mobile phase with a wide range of small-particle stationary phases offers a vast range of selectivity [11]. UPC^2, when coupled to highly sensitive tandem mass spectrometry becomes a powerful tool for separating and quantifying trace levels of analytes such as complex steroid hormones. Recent advances in mass spectrometry such as the inclusion of measures maximising ion transmission to the detector while removing neutral interferences, has led to increases in sensitivity by increasing the signal to noise (S/N) ratio. Our laboratory has clearly demonstrated the resolving power of UPC^2 when applied to steroid metabolites [14].

The aim of this study was to develop and validate a method, utilizing UPC^2-MS/MS, for the separation and quantification of the classical androgens and structurally related 11-oxygenated C_{19} steroid hormones. The latter group of steroid metabolites were recently identified in the metabolism of 11β-hydroxyandrostenedione (11OHA4), a major C_{19} adrenal androgen, which has been implicated as a role player in castration resistant prostate cancer (CRPC) (Fig.1) [14–17]. Significantly, two of the resulting steroids, 11-ketotestosterone (11KT) and 11-ketodihydrotestosterone (11KDHT) have recently been shown to be potent androgens, comparable to testosterone and DHT, respectively [18]. As multiple routes from 11OHA4 to 11KDHT exist, it is vital that the individual enzymatic steps as well as the pathway as a whole is characterised. To this end the current method was developed which allows for *in vitro* assays to be performed at physiologically relevant concentrations. The resulting method not only demonstrates the analytical power achieved by the UPC^2-MS/MS system, but also provides a necessary tool to further elucidate the role of steroid metabolites in the development and progression of CRPC and other androgen dependent tumours.

2 Experimental section

2.1 Reagents

2.1.1 Steroid standards

Androstenedione (A4) and testosterone (T) were purchased from Sigma-Aldrich (Steinheim, Germany) and Fluka (Neu-Ulm, Germany), respectively. 11β-hydroxyandrostenedione (11OHA4), 11-ketoandrostenedione (11KA4), 11β-hydroxytestosterone (11OHT), 11-ketotestosterone (11KT), 5α-androstanedione (5α-

dione), epiandrosterone (epiAST), 5α-dihydrotestosterone (DHT), 11-ketodihydrotestosterone (11KDHT), 5α-androstane-3α,17β-diol (3α-adiol), 11β-hydroxyandrosterone (11OHAST), 11β-hydroxyepiandrosterone (11OHepiAST), 11-ketoandrosterone (11KAST) and 11-ketoepiandrosterone (11KepiAST) were purchased from Steraloids (Wilton, USA).

2.1.2 Production of steroids which are not available commercially

The steroids 11β-hydroxy-5α-androstanedione (11OH-5αdione), 11keto-5α-androstanedione (11K-5αdione), 11β-hydroxydihydrotestosterone (11OHDHT) and 11keto-5α-androstane-3α, 17β-diol (11K-3α-adiol) were prepared using HEK293 cells transiently transfected with steroid 5α-reductase type 1 (SRD5A1) or 3α-hydroxysteroid dehydrogenase type 3 (AKR1C2) as previously described [15].

2.2.3 Internal standards

Gestodene (GES) and drospirenone (DRSP) were purchased from Sigma-Aldrich (Steinheim, Germany). Cortisol-9, 11, 12, 12-d_4 (cortisol-d_4) and testosterone-1, 2-d_2 (T-d_2), progesterone-2, 2, 4, 6, 6, 17α, 21, 21, 21-d_9 (Prog-d_9) and 4-pregnen-17α-ol-3,20-dione-2, 2, 4, 6, 6, 21, 21, 21-d_8 (17OHProg-d_8) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

2.1.4 Solvents

FOODFRESH CO_2 was purchased from Afrox (Cape Town, South Africa). UHPLC grade methanol, formic acid, and tert-Methyl Butyl Ether (MTBE) were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 Preparation of standards and samples

Individual stock solutions of the fifteen commercially available steroids (A4, T, 11OHA4, 11KA4, 11OHT, 11KT, 5α-dione, epiAST, DHT, 3α-adiol, 11KDHT, 11OHAST, 11OHepiAST, 11KAST and 11KepiAST) were prepared in absolute ethanol (1 mg mL^{-1}) and stored at −20 °C until use. These individual stock solutions were later used to prepare two standard master mixes (1000 ng mL^{-1} and 1 ng mL^{-1}) in methanol, which contained all of the above mentioned steroids. These standard master mixes were subsequently used to prepare standards (1mL, 0.01– 250 ng mL^{-1}) by the addition of the appropriate volume of the standard master mix to either DMEM containing 1% penicillin-streptomycin and 10% fetal bovine serum (matrix) or 50% methanol (no matrix). Samples used for method validation (1 mL) were prepared by spiking the matrix (DMEM containing 1% penicillin-streptomycin and 10% fetal bovine serum), with the appropriate volume of the standard master mix.

2.3 Steroid extractions

Fifty microliters of an internal standard mixture containing 15 ng cortisol-d_4, 1.5 ng T-d_2, 15 ng 17OHP4-d_9, 15 ng P4-d_9, 12.4 ng GES and 14.7 ng DRSP was added to each sample and standard prior to extraction. Samples and standards were extracted using a 1:3 ratio of sample to MTBE (vol vol^{-1}). The samples were shaken at 1000 RPM for 15 min before being placed at -80°C for an hour to allow the aqueous phase to freeze. The MTBE layer containing steroids was transferred to a pyrolysed glass test tube and the MTBE evaporated at 55°C under a stream of nitrogen gas. Samples were subsequently reconstituted in 150 µL 50% methanol and stored at −20°C prior to analysis.

2.4 Instruments and chromatographic conditions

Steroid metabolites were separated using an Acquity UPC^2 system (Waters Corporation, Milford, USA) with an Acquity UPC^2 BEH 2-EP column (3 mm X 100 mm, 1.7 µm particle size). The mobile phase consisted of liquid CO_2 modified with methanol. A 4 min linear gradient from 2% to 9.5% methanol was used to separate the C_{19} steroids using a constant flow rate of 2.0 mL min^{-1}. The column temperature and automated back pressure regulator (ABPR) were set to 60°C and 2000 psi, respectively. The injection volume was 2.0 µL. Quantitative mass spectrometric detection was carried out using a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA). A make-up pump was attached to this coupler which fed 1% formic acid in methanol into the mixer preceding the MS line at a constant flow rate of 0.2 mL min^{-1}. All steroids were analysed in multiple reaction monitoring (MRM) mode using an electrospray probe in the positive ionization mode (ESI+). The following settings were used: capillary voltage of 3.8 kV, source temperature 120°C, desolvation temperature 500°C, desolvation gas 1000 L h^{-1} and cone gas 150 L h^{-1}. MRM settings are included in table1. Data collection and analysis were performed using MassLynx 4.1 (Waters Corporation).

2.5 Method validation

Standard curves were generated for each steroid metabolite using standards prepared in matrix or 50% methanol and included the following concentrations: 0, 0.01, 0.05, 0.1, 0.25, 0.5, 1.0, 5.0, 10, 25, 50, 100 and 250 ng mL^{-1}. The limit of detection (LOD) for each steroid metabolite was defined as the lowest concentration at which a signal-to-noise (S/N) ratio greater than 3 was measured for the quantifier ion. The limit of quantification (LOQ) was defined as the lowest concentration for each steroid at which a S/N ratio greater than 10 was measured for quantifier ion, and a S/N ratio greater than 3 measured for the qualifier ion, and which could be

measured with acceptable precision (≤20%) expressed as percent relative standard deviation (%RSD). The upper limit of quantification was defined as the highest concentration for each steroid which fit a linear standard curve, and which could be measured with acceptable precision (≤15%) expressed as percent relative standard deviation (%RSD). Linearity of the standard curves was assessed using the Runs test in Graphpad Prism. Accuracy and precision were determined for the following concentrations: 0.25, 2.5, 25, 50 and 100 ng mL^{-1}.Accuracy was defined as the %RSD from the average calculated concentration following the repeated injection (n=8) of a single sample. Precision was defined as the %RSD from the analysis of independent replicate samples (n=6) and was repeated over three days in order to assess inter day variation. Precision was also used to assess within-laboratory reproducibility for two assessors over four days for the concentrations 25 and 100 ng mL^{-1}. Recovery, matrix effect and process efficiency were determined at three concentrations (2.5, 50 and 100 ng mL^{-1}, n=3) for each steroid using the method described by Taylor *et al* 2005 [19]. Briefly, steroid recovery was calculated by comparing the response of standards prepared in media and extracted as described above to that of media extracts with the post-extraction addition of steroids. Similarly matrix effects were determined by comparing media extracts with the post-extraction addition of steroids to a pure solution of 50% methanol containing equivalent amounts of steroid. The difference in response between the post-extraction samples and the pure standards were divided by the response of the pure standards. The overall process efficiency, which is the combination of recovery and matrix effects, was calculated by comparing the response of standards prepared in media (pre-extraction) to that of pure standards.

2.6 Comparative separation and quantification by UPLC-MS/MS

Steroid metabolites were separated using a UPLC hollow structural section (HSS) T3 column (2.1 mm x 50 mm, 1.8 µm) (Waters, Milford, USA) coupled to an ACQUITY UPLC (Waters, Milford, USA) as previously described [4]. The mobile phases consisted of (A) 0.1% formic acid and (B) 100% methanol. A 5 min linear gradient from 55% A to 75% B was used to separate the C_{19} steroids using a constant flow rate of 0.6 mL min^{-1} and a column temperature of 50°C. The injection volume was 20.0 µL. Quantitative mass spectrometric detection was carried out using a Xevo TQ triple quadrupole mass spectrometer (Waters, Milford, USA). All steroids were analysed in multiple reaction monitoring (MRM) mode using an electrospray probe in the positive ionization mode (ESI+). The following settings were used: capillary voltage of 4.0 kV, source temperature 150°C, desolvation temperature 500°C, desolvation gas 1000 L h^{-1} and cone gas 150 L h^{-1}. Data collection and analysis were performed using MassLynx 4.1 (Waters Corporation).

3 Results

3.1 Separation by UPC^2

We have developed an UPC^2-MS/MS which can be used to identify nineteen and quantify fifteen C_{19} steroids, including all 11OHA4 pathway metabolites, in 5 min using a 1.7 µm particle size BEH 2-EP column (Fig. 2). The separation by UPC^2 was achieved using a flow rate of 2.0 mL.min^{-1} and ABPR of 2000 PSI. These parameters ensured high chromatographic efficiency (supplementary table 1) and high throughput, while maintaining an acceptable system backpressure. The separation includes four novel steroids which we have identified in the 11OHA4 pathway, but are not commercially available [15,16]. Although 11KepiAST and

11KDHT as well as 3α-adiol and 11OHAST were not resolved these steroids can be quantified using their unique MRMs. We found that the separation achieved by UPC^2 was orthogonal to that of reverse-phase LC as the elution order was not simply the reverse of that obtained by reverse-phase UPLC, even though normal phase column chemistries were employed (Fig. 3). For example the separation of six closely related steroids, 11OHA4, 11KA4, 11OHT, 11KT, A4 and T, by conventional reverse-phase UPLC resulted in two distinct groupings based on polarity (Fig. 3.a), with A4 and T being the least polar. The separation of the same six steroids by UPC^2 did not demonstrate the same groupings, but instead yielded a mixed elution order (Fig. 3.b). The altered selectivity achieved by UPC^2 is further illustrated in Fig. 4 in which the elution order of nineteen C_{19} steroids separated by reverse-phase UPLC is plotted against to the elution order obtained by the UPC^2 method.

3.2 Mass Spectrometry

The UPC^2 eluent was mixed with the makeup fluid to ensure the ionization of the steroids by ESI. The flow rate and the composition of makeup fluid were optimized with respect to the ionization efficiency of steroids (the makeup fluid is required for the ionization). The optimal flow rate was found to be 0.2 mL min^{-1}. Methanol containing 1% formic acid provided good ionization efficiency for the steroid metabolites. The ionization and fragmentation behaviour of individual steroids obtained on the UPC^2-MS/MS system is the same as that obtained by UPLC-MS/MS (table 1). Apart from a few exceptions, the most abundant ions obtained in positive-ion mode are protonated species $[M + H]^+$. The most abundant ions obtained for AST, epiAST, 11OHAST and 11OHepiAST were $[M – H_2O + H]^+$.

3.3 Performance and Validation

3.3.1 Calibration range

Comprehensive method validation was performed for the 15 steroids which are available commercially (table 2). LOQs ranged from 0.01 to 10 ng mL^{-1}, demonstrating the ionization efficiencies of the different steroids. A 50 fold increase in sensitivity for steroids which ionize easily, such as T and A4, was observed when comparing a Waters Acquity UPLC coupled to a Xevo TQ mass spectrometer to the UPC^2 system coupled to the Xevo TQ-S mass spectrometer. We also observed significant improvements in the LOQ's for difficult to ionize steroids, such as DHT (5 fold). These improvements were despite a 10-fold lower injection volume. The LOQs achieved using the UPC^2-Xevo TQ-S system allow for the quantification of steroids at levels which were previously not possible. The current method can also be used for the detection and relative quantification of the novel steroids 11OH-5α-dione, 11K-5α-dione, 11OHDHT and 11K-3α-adiol. As these steroids are not commercially available they could not be accurately quantified and therefore were excluded from comprehensive validation. The calibration range achieved with the Xevo TQ is linear up to a concentration of 500 ng mL^{-1}, but only 100 ng mL^{-1} when using the Xevo TQ-S, due to the improved sensitivity of the latter system (table 2). Quadratic fits with acceptable r^2-values (> 0.9906) were obtained for standard curves which included a 250 ng mL^{-1} standard, however, due to saturation of the detector precision and accuracy were both greater than 20% at this concentration and this standard was therefore excluded from the calibration range. The upper limit of quantification (ULOQ) was therefore 100 ng mL^{-1}.

3.3.2 Accuracy and precision

Accuracy and precision were determined at a minimum of three concentrations within the calibration range of each steroid and are shown in tables 1 and 2, respectively. Acceptable RSDs were obtained for all concentrations for both accuracy and precision, which was determined across three days (table 3) [20]. Both accuracy and precision were less than 15% at concentrations of 50 and 100 ng mL^{-1} (medium and high). Accuracy at low concentrations ranged from 10 to 17% at 0.25 ng mL^{-1}, 7 to 20% at 2.5 ng mL^{-1} and 8 to 20% at 25 ng mL^{-1}. Precision at low concentrations ranged from 1 to 14% at 0.25 ng mL^{-1}, 4 to 19% at 2.5 ng mL^{-1} and 2 to 15% at 25 ng mL^{-1}. Within-laboratory reproducibility is shown in supplementary table 2.

3.3.3 Recovery, matrix effect and process efficiency

Recovery, matrix effect and process efficiency are shown in table 4. Recovery ranged from 54.7 to 78.1% at the low concentration. Recovery values between 74.9 – 121.1% were obtained at medium and high concentrations (50 and 100 ng mL^{-1}). Matrix effects ranged from 18.7% ion suppression for 11KDHT to 20.8% ion enhancement for 11KepiAST). The average process efficiency, which is a combination of recovery and matrix effect, ranged from 55.4 to 78.5% at a concentration of 2.5 ng mL^{-1} and 73.6 to 114.7% at concentrations of 50 and 100 ng mL^{-1}.

4 Discussion

We have developed a novel UPC^2-MS/MS method to study androgen metabolism. This method identifies 19 steroids including those in the newly identified 11OHA4 pathway in a high-throughput method of less than 5 min. Separation by UPC^2 was

superior to that achieved by reversed phase UPLC based on superior selectivity and increased chromatographic efficiency (supplementary table 1). The selectivity obtained by UPC^2 resulted in a scattered elution order compared to that of the UPLC (Fig. 3, Fig. 4) [8,15]. This exceptional selectivity is ideal for the chromatographic separation of compounds such as steroids, which have both similar structures and mass spectra.

The sensitivity of the developed method is also superior (5-50 times improvement in signal) to any current available methods (without derivatization). The increased sensitivity is due to a combination of the optimal flow rate achieved by setting the make-up pump [21–23] and the reduction of interfering neutral contaminants by the StepWave ion guide [24]. The only drawback to the increased sensitivity is the reduced linear range due to saturation of the detector at higher concentrations. This is, however, easily overcome by further sample dilution. The increased sensitivity for the first time allows us to investigate steroid metabolism in cell culture at physiological concentrations. For example we have been able to measure the metabolism of 10 nM 11KT and T by prostate cancer cell lines [18]. Such experiments have previously relied on the use of supraphysiological substrate concentrations due to limitations in sensitivity. Moreover, increased sensitivity is achieved using injection volumes of only 2 µL, while conventional UPLC-MS/MS methods rely on injection volumes of 5 to 20 µL in order to achieve satisfactory sensitivities. The reduced injection volume is a significant advantage when working with biological samples in which the amount of sample is limited. Alternatively the reduced injection volume allows for multiple injections to be completed from the same sample. All other parameters, such as accuracy, precision and matrix effects were equivalent to those achieved by UHPLC-MS/MS.

5 Conclusion

The current method has been developed specifically as a tool to study the role of androgen metabolism in the development and progression of CRPC, but also serves to demonstrate the increased selectivity and sensitivity which can be achieved by UPC^2-MS/MS over conventional UPLC-MS/MS. The timing of this new technology is opportune as there is currently a drive within the field of endocrinology to phase out the use of immunoassays in favour of validated MS-based assays [25–27]. This method has clearly demonstrated that the selectivity and reduced run times achieved by UPC^2 are ideal for both clinical and research settings as they allow for the simultaneous quantification of numerous steroid metabolites, while at the same time achieving high throughput. Supercritical CO_2 is also inexpensive when compared to organic solvents and has a significantly reduced environmental impact [11]. The inclusion of the latest MS technology which incorporates steps to reduce neutral contaminants in combination with the UPC^2 inlet system results in increases in sensitivity which are ideal for the quantification of physiological levels of steroid hormones from complex biological matrices.

The sensitivity and selectivity achieved by this method makes it ideally suited for multiple *in vitro* and *in vivo* applications, such as investigations into CRPC, testicular, breast, ovarian, colon, endometrial, adrenal and other hormone dependent cancers, where the role of these novel androgens is unclear. The quantification range is ideal for the use in cell culture, xenograft, tissue, serum and plasma samples. While to utility of this method for hormone related cancers is apparent, other applications can be imagined such as in patients with disorders of sexual differentiation (DSD) where altered steroid metabolism is observed. It is possible that there is a previously undiscovered role for these androgens in patients with DSD and further research is

needed to elucidate this. This method therefore unlocks possibilities for new applications which can benefit from the enhanced separation and detection offered by UPC^2-MS/MS.

6 Conflict of Interest Disclosure.

The authors declare no competing financial interest.

7 Acknowledgements

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Figure 1. Schematic representation of the 11OHA4 pathway. 3αHSD, 3α-hydroxysteroid dehydrogenase; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2; 11K-3α-adiol, 11-keto-3α-androstanediol; 11K-5α-dione, 11-keto-5α-androstanedione; 11KA4, 11-ketoandrostenedione; 11KAST, 11-ketoandrosterone; 11KDHT, 11-keto-5α-dihydrotestosterone; 11KT, 11-ketotestosterone; 11OH-5α-dione, 11β-hydroxy-5α-androstanedione; 11OHA4, 11β-hydroxyandrostenedione; 11OHAST, 11β-hydroxyandrosterone; 11OHT, 11β-hydroxytestosterone; 17βHSD2, 17β-hydroxysteroid dehydrogenase type 2; AKR1C3, aldo-keto reductase 1C3; SRD5A1, steroid 5α-reductase type 1.

Figure 2. UPC^2-MS/MS chromatogram depicting the elution order of C_{19} steroids. The steroids 5α-dione (1), A4 (2), 11K-5α-dione (3), 11KA4 (4), DHT (5), epiAST (6), 11OH-5α-dione (7), T (8), 11KAST (9), 11OHA4 (10), 11KepiAST (11), 11KDHT (12), 3α-adiol (13), 11OHAST (14), 11KT (15), 11OHepiAST (16), 11OHDHT (17), 11K-3α-adiol (18) and 11OHT (19) were separated by UPC^2 (ACQUITY UPC^2, Waters, Milford, USA) using an ACQUITY UPC^2 BEH 2-EP (3.0 mm X 100 mm, 1.7 µm) column.

Figure 3. Chromatographic separation of 11KA4 (1), 11KT (2), 11OHA4 (3), 11OHT (4), A4 (5) and T (6) by (a) UPLC-MS/MS and (b) UPC^2-MS/MS.

Figure 4. Graphic depicting the orthogonal elution order of androgens by UPLC and UPC^2.

Table 1. Molecular ion species, MRM mass transitions, mass spectrometer parameters (cone voltage, collision energy) and retention time for each steroid. Internal standard: PROG-d_9, 17OHPROG-d_9, T-d_2, Cortisol-d_4, DRSP and GES.

		Mass transitions						
Abbreviation	Name	Quantifier	Qualifier	Cone Voltage (V)		Collision Energy (eV)		Retention Time (min)
11OHA4	11β-hydroxyandrostenedione	303.2>267.2	303.2>121.0	30	30	30	15	2.20
11KA4	11-ketoandrostenedione	301.2>257.0	301.2>265.2	35	35	25	25	1.30
11OHT	11β-hydroxytestosterone	305.3>121.0	305.3>269.0	35	35	20	15	3.79
11KT	11-ketotestosterone	303.2>121.0	303.2>267.0	30	30	20	20	2.70
11OH-5α-dione	11β-hydroxy 5α-androstanedione	305.0>269.2	305.0>287.2	35	35	15	15	1.52
11K-5α-dione	11-keto-5α-androstanedione	303.2>241.0	303.2>267.0	35	35	30	25	0.90
11OHDHT	11β-hydroxy-5α-dihydrotestosterone	307.0>253.0	307.0>271.0	35	35	20	20	3.12
11KDHT	11-ketodihydrotestosterone	305.2>243.0	305.2>269.0	30	30	20	20	2.46
11OHAST	11β-hydroxyandrosterone	289.0>271.0	289.0>213.0	15	15	15	15	2.62
11OHepiAST	11β-hydroxyepiandrosterone	289.0>271.0	289.0>213.0	15	15	15	15	2.88
11KAST	11-ketoandrosterone	305.0>147.2	305.0>173.1	30	30	30	30	2.04
11KepiAST	11-ketoepiandrosterone	305.0>147.2	305.0>173.1	30	30	30	30	2.46
11K-3α-adiol	11-keto 5α-androstane-3α,17β-diol	307.2>271.0	307.2>253.0	15	15	10	10	3.56
A4	Androstenedione	287.2>96.9	287.2>108.8	30	30	15	15	0.88
Testosterone	Testosterone	289.2>97.2	289.2>109.0	30	30	22	22	1.86
5α-dione	5α-androstanedione	289.2>253.1	289.2>97.2	22	30	16	22	0.57
DHT	5α-dihydrotestosterone	291.2>255.0	291.2>273.0	25	25	15	20	1.38
epiAST	Epiandrosterone	273.2>105.4	291.3>273.4	30	18	30	8	1.45
3α-adiol	5α-androstane-3α,17β-diol	275.2>257.0	275.2>175.0	15	15	15	15	2.62
PROG-d_9	Progesterone-2, 2, 4, 6, 6, 17α, 21, 21, 21-d_9	324.2>100.0	324.2>113.0	30	30	20	25	0.84
17OHPROG-d9	4-Pregnen-17α-ol-3,20-dione-2, 2, 4, 6, 6, 21, 21, 21-d_8	340.1>100.0	340.1>114.0	26	26	25	28	1.75
T-d_2	Testosterone-1, 2-d_2	291.0>99.1	291.0>111.2	30	30	20	30	1.86
Cortisol-d_4	Cortisol-9, 11, 12, 12-d_4	367.0>121.0		35		25		4.24
DRSP	Drospirenone	367.2>97.3	367.2>159.3	20	20	30	25	2.24
GES	Gestodene	311.2>109.4	311.2>135.0	15	15	25	25	1.62

Table 2. Comprehensive method validation data: LOD (ng mL^{-1}); LOQ (ng mL^{-1}); Range (ng mL^{-1}); r^2; Accuracy (RSD %, n= 8).

Steroids	LOD (ng mL^{-1}) (pg on column)	LOQ (ng mL^{-1}) (pg on column)	Range (ng mL^{-1})	r^2	Accuracy RSD % (ng mL^{-1})				
					0,25	2,5	25	50	100
11OHA4	0.01 (0.13)	0.01 (0.13)	0.01 - 100	0.9917	16.0%	7.0%	11.5%	5.1%	2.6%
11KA4	0.01 0.13)	0.01 (0.13)	0.01 - 50	0.9977	17.5%	13.3%	12.0%	13.9%	9.1%
11OHT	0.01 (0.13)	0.01 (0.13)	0.01 - 100	0.9946	14.0%	10.0%	8.4%	9.3%	6.0%
11KT	0.01 (0.13)	0.01 (0.13)	0.01 - 100	0.9989	13.1%	14.6%	8.6%	5.3%	5.9%
11KDHT	0.25 (3.33)	1 13.3)	0.25 - 100	0.9996	<LOQ	15.0%	9.2%	7.1%	6.0%
11OHAST	5 (66.7)	10 (133.3)	10 - 100	0.9985	<LOQ	<LOQ	11.7%	9.4%	13.0%
11OHepiAST	5 (66.7)	10 (133.3)	10 - 100	0.986	<LOQ	<LOQ	20.3%	9.5%	12.0%
11KAST	5 (66.7)	5 66.7)	5 - 100	0.9958	<LOQ	<LOQ	11.4%	6.5%	8.4%
11KepiAST	10 (133.3)	10 (133.3)	10 - 100	0.9951	<LOQ	<LOQ	18.5%	7.6%	8.0%
A4	<0.01 (<0.13)	0.01 (0.13)	0.01 - 100	0.9851	13.4%	9.3%	7.5%	5.4%	9.0%
T	<0.01 (<0.13)	0.01 (0.13)	0.01 - 50	0.992	10.0%	20.1%	8.4%	4.0%	4.9%
5α-dione	0.25 (3.33)	0.5 (6.67)	0.5 - 100	0.9941	<LOQ	16.5%	18.5%	9.7%	7.7%
DHT	0.25 (3.33)	1 (13.3)	1 - 100	0.9942	<LOQ	13.0%	12.4%	11.3%	9.7%
epiAST	0.25 (3.33)	1 (13.3)	1 - 100	0.9952	<LOQ	15.1%	9.9%	6.0%	10.0%
3α-adiol	1 (13.3)	5 (66.7)	5 - 100	0.9977	<LOQ	<LOQ	13.5%	7.2%	11.5%

Table 3. Comprehensive method validation data continued: Precision with interday variation (RSD %, n= 6).

Steroids	Precision RSD %														
	Day 1					Day 2					Day 3				
	Concentration (ng mL^{-1})														
	0.25	2.5	25	50	100	0.25	2.5	25	50	100	0.25	2.5	25	50	100
11OHA4	9.8%	3.9%	8.1%	3.6%	2.8%	7.6%	19.1%	7.5%	6.5%	8.4%	14.2%	12.6%	8.5%	5.0%	2.9%
11KA4	11.8%	11.7%	15.1%	1.9%	2.9%	9.8%	11.7%	2.0%	3.9%	5.0%	8.8%	12.5%	3.8%	4.9%	3.5%
11OHT	2.6%	15.2%	4.5%	5.4%	10.2%	9.5%	11.8%	8.1%	5.7%	7.4%	12.5%	11.4%	7.0%	6.7%	3.8%
11KT	4.4%	8.3%	6.5%	4.0%	3.7%	8.7%	7.0%	9.5%	6.5%	7.2%	10.9%	12.8%	6.6%	6.0%	5.6%
11KDHT	<LOQ	13.5%	8.4%	3.4%	3.7%	<LOQ	11.8%	8.0%	5.4%	9.1%	<LOQ	4.7%	5.3%	7.9%	13.3%
11OHAST	<LOQ	<LOQ	8.2%	5.0%	6.7%	<LOQ	<LOQ	10.2%	4.9%	6.1%	<LOQ	<LOQ	11.3%	5.7%	3.6%
11OHepiAST	<LOQ	<LOQ	11.3%	5.1%	2.7%	<LOQ	<LOQ	5.5%	2.8%	8.7%	<LOQ	<LOQ	5.6%	4.2%	5.1%
11KAST	<LOQ	<LOQ	10.4%	3.5%	3.5%	<LOQ	<LOQ	4.6%	3.6%	6.1%	<LOQ	<LOQ	6.8%	3.4%	4.0%
11KepiAST	<LOQ	<LOQ	13.6%	5.8%	2.9%	<LOQ	<LOQ	8.5%	7.9%	6.4%	<LOQ	<LOQ	14.2%	12.5%	9.5%
A4	5.2%	9.7%	5.4%	2.6%	4.4%	4.2%	11.3%	2.9%	3.3%	4.0%	4.3%	8.0%	4.2%	4.1%	4.2%
T	3.2%	9.5%	6.5%	2.4%	2.4%	1.2%	11.2%	2.1%	4.7%	2.9%	5.0%	11.0%	3.9%	4.4%	4.2%
5α-dione	<LOQ	9.8%	6.2%	2.6%	5.6%	<LOQ	15.0%	9.2%	8.1%	5.8%	<LOQ	15.5%	6.3%	5.0%	4.8%
DHT	<LOQ	10.5%	8.8%	2.9%	2.2%	<LOQ	9.6%	6.4%	6.2%	5.9%	<LOQ	9.8%	3.2%	5.7%	6.2%
epiAST	<LOQ	14.7%	12.3%	7.6%	3.9%	<LOQ	15.5%	10.8%	8.9%	6.5%	<LOQ	9.5%	5.6%	4.6%	8.5%
3α-adiol	<LOQ	<LOQ	9.2%	11.6%	2.3%	<LOQ	<LOQ	5.4%	6.6%	6.5%	<LOQ	<LOQ	9.9%	6.7%	9.8%

Table 4. Comprehensive method validation data continued, Recovery (%, n=3); Matrix Effect (%, n=3) and Process Efficiency (%, n=3).

Steroids	Recovery % ng mL^{-1}			Matrix effect % ng mL^{-1}			Process Efficiency % ng mL^{-1}		
	2,5	**50**	**100**	**2,5**	**50**	**100**	**2,5**	**50**	**100**
11OHA4	63.1	109.0	106.0	8.9	-8.0	-12.4	68.7	100.2	92.8
11KA4	77.8	101.0	105.5	-19.0	-1.0	-16.9	63.0	100.0	87.6
11OHT	64.7	109.1	109.6	13.3	5.2	-10.6	73.4	114.7	98.0
11KT	56.4	83.6	80.4	1.9	1.7	6.8	57.4	85.1	85.9
11KDHT	78.1	121.1	108.6	-14.2	-16.6	-18.7	67.0	101.0	88.3
11OHAST	<LOQ	85.8	88.7	35.3	-0.8	-3.5	65.0	85.1	85.6
11OHepiAST	<LOQ	96.0	88.0	40.1	17.7	13.8	97.5	113.1	100.1
11KAST	<LOQ	106.8	94.2	3.7	-6.2	-2.2	86.8	100.2	92.1
11KepiAST	<LOQ	78.1	87.1	7.5	20.8	0.6	73.2	94.4	87.6
A4	62.9	89.6	74.9	2.8	-3.2	-1.7	64.7	86.7	73.6
T	62.0	103.0	90.8	11.0	-1.8	1.1	68.8	101.1	91.9
5α-dione	55.7	112.4	85.6	-0.4	-15.6	-4.2	55.4	94.9	82.0
DHT	64.0	102.2	112.6	-5.9	-9.1	-17.1	60.2	92.9	93.4
epiAST	72.5	102.0	114.8	8.4	-1.0	-13.3	78.5	100.9	99.5
3α-adiol	54.7	92.3	119.0	28.4	11.3	-12.8	70.2	102.6	103.8

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