Ultra-Performance Supercritical Fluid Chromatography Hyphenated to Tandem Mass Spectrometry for a Novel Targeted Analysis of Peripheral Steroids

[1]Kondepudi Lalitha, [2]Sanikommu Kalpana, [3]Saripalli Sri Lakshmi*

[1,2]Student, [3]Assistant professor
Department of Pharmaceutical Analysis,
Jawaharlal Nehru Technological University JNTUK,
School of Pharmaceutical Sciences and Technologies
Kakinada, Andhra Pradesh, India-533003

***Abstract*- An alternate technique for steroid analysis is ultra-performance supercritical fluid chromatography-tandem mass spectrometry (UPSFC-MS/MS). In order to deliver relevant and thorough results, it is crucial that analytical procedures for steroid profiling are continuously improved. This study's objective was to rapidly and accurately detect and measure a large number of endogenous steroids from the four main classes (oestrogens, androgens, progestogens, and corticosteroids). This brand-new UPSFC-MS/MS technique uses electrospray in positive ionization (ESI+) mode, which is robust, selective, and sensitive enough to profile 19 steroid compounds in 50 liters of human plasma. In the multiple reaction monitoring (MRM) mode, 19 distinct steroids were successfully isolated under optimal conditions. With correlation coefficients (R^2) in the range of 0.9883 to 0.9999 and a calibration range of 0.05 to 500 ng/mL in human plasma, the method's linearity was good. The method's intraday and intraday precision, measured as RSD, was under 15%. The nineteen analytes' accuracy ranged from 80 to 116%. Finally, the innovative technology was successfully used to identify nineteen steroids in under five minutes, opening the door for their potential usage in both ordinary medical treatment and research**

***keywords*— UPSFC-MS/MS, ESI+, MRM, APCI, APPI, BEH, CSH Fluorophenyl**

I. INTRODUCTION

Endogenous steroids, which include estrogens, androgens, progestogens, corticosteroids, and their metabolites, are physiologically significant substances that are produced spontaneously in the body and regulate a variety of bodily processes. These substances come from cholesterol, which is primarily created from scratch in all animal and plant cells, including human cells. In various organs, such as the brain, adrenal glands, gonads, and placenta, steroids are created during the steroidogenesis of cholesterol.

The use of steroids as potential biomarkers in medical practise has received more attention over the past 20 years. It is well known that steroid hormone production declines with age and has been linked to a number of endocrine and metabolic disorders.

The analytical techniques based on tandem mass spectrometry and chromatography for identifying steroids in biological samples have recently attracted a lot of attention. An important source of knowledge on several illnesses is steroid profiling in regular clinical diagnosis. Due to the tiny concentration levels in various biological samples, a precise measurement of steroids in biological tissues has therefore become crucial for modern medicine, even though it is challenging.

The quantification of steroids employs a variety of methods. Immunoassays, such as radioimmunoassay or enzyme immunoassay, are the most frequently used techniques for steroid measurement in clinical practise. The primary drawbacks of immunoassay methods include their susceptibility to matrix effects and the cross- reactivity of the assay's antibodies with the relevant steroids. Most contemporary separation techniques for two or more steroids rely on tandem mass spectrometry (MS/MS) in combination with either liquid chromatography (LC) or gas chromatography (GC). These techniques provide simultaneous determination of the four main classes of steroids (oestrogens, androgens, progestogens, and corticosteroids), and they offer valuable information in the clinical setting. Additionally, these high-tech techniques are incredibly valuable for acquiring structural data on specific steroids and their metabolites.

A variety of chemical derivatization techniques are frequently used in conjunction with GC-MS analysis of steroids and their metabolites in biological samples. In order to increase the sensitivity of the steroid analysis, GC has been hyphenated with numerous different types of mass spectrometers, including the triple quadrupole (TQ) and tandem Mass. Similarly, LC has been connected to many MS systems, with the most popular ionisation methods being air pressure chemical ionisation (APCI) and electrospray ionisation (ESI). The use of LC-MS/MS for the analysis of steroids without derivatization in clinical practise is well established. Compared to GC-MS/MS, which offers superior chromatographic resolution, the advantages of LC-MS/MS include less sample preparation and quicker analytical times.

Supercritical fluid chromatography (SFC) is a crucial analytical method utilised for rapid and extremely effective separation. SFC has recently undergone improvements that make it an effective method for analysing a variety of chemicals, including non-polar, polar, and ionisable analytes. SFC can display a variety of chromatographic behaviours, including ion pairing, normal-phase, reverse-phase, and combinations of these various modes. Due to the major mobile phase's decreased viscosity, quick and high resolution separations are possible at reasonable pressures (CO2). A stationary phase to provide adequate resolution and the addition

of a suitable co-solvent for analyte solvation are essential components of the creation of an SFC technique. In addition, compared to other separation methods, SFC enhances the separation of isomers and enantiomers. Therefore, integrating SFC with MS/MS offers a number of benefits in terms of sensitivity and specificity. We believe that there is currently no UPSFC-MS/MS (UP signifies ultra-performance) technology available for the simultaneous quantification of endogenous hormones from the four main classes in tiny amounts of human plasma. With the use of our technique, the levels of 19 endogenous steroid hormones can be quickly and accurately determined in 50L of plasma

II. UPSFC MS/MS ACQUISITION

Using a UPSFC-MS/MS technique, distinct endogenous steroid hormones and metabolites were successfully separated in less than 5 minutes. The unique aspect of the current study is the development of a quick, sensitive, and trustworthy approach for quantifying endogenous hormones simultaneously in all four major steroid classes. The majority of methods for steroid analysis that have been disclosed are concentrated on identifying a small number of steroids within one or two classes. Only 12.5% of all published papers' authors, according to a recent review, stated they were simultaneously analysing 8 to 35 steroid analytes from all four major classes using GC-MS/MS or LC-MS/MS techniques.

As far as we are aware, very few studies have used SFC-MS/MS to analyse steroids and their metabolites. With a 10 minutes separation time, Xu et al. examined the standards of the estrogenic class and its metabolites. Before analysis, these steroids underwent dansyl chloride derivatization. Two columns made up the chromatography setup: a diol column for two-dimensional studies and a cyano-propyl silica column coupled in series. This approach was used to simply look for oestrogen metabolites in the analysis. Estrone (E1) elutes in our approach at 2.17 minutes, whereas it did so the other way at 5.25 minutes. A BEH was developed for a high-throughput study of underivatized androgenic steroids by Quanson et al. in a more recent paper. The analysis of eight glucuronide and ten sulphate steroids from the estrogenic and androgenic classes in urine was also reported by Doué et al. using a new SFC-MS/MS technique. A BEH column and a BEH 2-EP column were used, respectively, to fractionate and separate glucuronide and sulphate steroids. Each separation was completed in 8 minutes. Parr et al. reported the analysis of 32 underivatized steroids with an analysis time of 21 min and LODs ranging from 1 to 50 ng/mL in their most recent publication. In contrast, our approach is quick (5 minutes), and for the majority of the steroids we examined, the LODs were less than 0.05 ng/mL.

As ionisation sources for MS detection, UPSFC has been coupled to ESI, APCI, and atmospheric pressure photoionization (APPI). According to Parr et al., ESI+ is preferable for a steroid mixture. However, the lack of readily ionisable moieties in the steroid molecule restricts the use of ESI-MS in steroid analysis. More specifically, the hydroxyl and carbonyl groups in steroid compounds are functional groups with low proton affinities. To raise the ionisation capacity by protonating or deprotonating the steroid molecule, which significantly boosts the analytes' ionisation efficiency (IE), chemical addition to these functional groups in the steroid ring is required. To improve the IE of steroid analytes, there are a number of derivatization techniques. Here, we used the carbonyl-reacting methoxyamin (MO).

Due to the greater proton affinity of the nitrogen-containing moiety in the resulting oxime derivatives, steroid analytes are more easily detected, and their IE has improved. The fragmentation patterns improve the detection of steroids by increasing sensitivity and selectivity. Additionally, the derivatization produced two isomers for each of the eleven steroids (aldosterone, androstenedione, testosterone, dihydrotestosterone, progesterone, 17-hydroxyprogesterone, cortisone, cortisol, and corticosterone), and both peaks were utilised to quantify each of the eleven steroids. Due to steric hindrance, the 11-keto group did not respond to the derivatization conditions. Baseline separation can be seen in the corresponding peaks of the geometric syn- and anti-isomers of oximes. Additionally, prior to disclosing the results, we determined that 45 minutes at 60 degrees was the ideal incubation time for the MO derivatization.

IntelliStart™ infusion mode was used to optimise the mass spectrometric settings. For all 19 steroids, using ESI in positive mode produced the greatest results. The IE was improved by methanol when 0.1% formic acid was used as a make-up solvent, notably for the chemicals that eluted at the start of the analysis, likely as a result of the creation of a stable spray. For each analyte, the $[M + H]^+$ ion was chosen as the precursor ion, and the greatest intensity product ion or ions were chosen to build the MRM technique. For each steroid analyte, the collision energies for the MRM transitions were optimised, and they are presented.

On UPSFC, the choice of stationary phase significantly affects the selective separation of analytes. For the initial screening of the steroids, three distinct stationary phases—BEH, BEH 2-EP, and CSH fluorophenyl (3.0 mm, 100 mm, 1.7 m)—were utilised. Each steroid's peak form and resolving power were assessed. CO2 (A) and 0.1% formic acid in methanol-isopropanol (1:1) as co-solvents made up the mobile phase (B). The general screening gradient increased linearly from 2% B to 17% B in 2 minutes. BEH provided The best peak shapes and resolution of the isomeric/isobaric pairs of steroids, including testosterone/dehydroepiandrosterone, androsterone/etiocholanolone,17-hydroxyprogesterone/11-deoxycorticosterone, and corticosterone/11-deoxycortisol, making it the most promising stationary phase, according to preliminary results. The BEH column was therefore chosen for additional analysis.

The selection of the stationary phase has a substantial impact on the selective separation of analytes on UPSFC1. Three different stationary phases—BEH, BEH 2-EP, and CSH fluorophenyl (3.0 mm, 100 mm, 1.7 m)—were used for the initial screening of the steroids. The peak shape and resolving power of each steroid were evaluated. The mobile phase consisted of CO2 (A) and 0.1% formic acid in methanol-isopropanol (1:1) as a co-solvent (B). In two minutes, the overall screening gradient went from 2% B to 17% B linearly. According to preliminary results, BEH was the most promising stationary phase, providing the best peak shapes and resolution of the isomeric/isobaric pairs of steroids, including testosterone/dehydroepiandrosterone, androsterone/etiocholanolone, 17-hydroxyprogesterone/11-deoxycorticosterone, and corticosterone/11-deoxycortisol. This led to the decision to conduct additional research on the BEH column.

The solubility of derivatized steroids is increased by the inclusion of additives (acids or bases) at low concentrations in the mobile phase, which produces more symmetric peak shapes. Investigations were conducted on six co-solvent formulations with and without

additions. We tested methanol and methanol, isopropanol, and/or acetonitrile mixes. In addition, formic acid and ammonium hydroxide were used.

On a BEH column, the separation of 19 steroids and their matching internal standards was accomplished in the current investigation successfully and quickly. Strong ionic interactions between free silanol groups that are present at the surface of this stationary phase may be the cause of the retention of basic oxime derivative analytes on the polar BEH stationary phase. An increase in polarity should lengthen the retention time of analytes in polar stationary phases like BEH, whereas molar volume has the opposite effect. For instance, cortisol, which is more polar than cortisone, elutes later, and steroids from all four classes follow a similar trend.

The co-solvent with 0.1% formic acid in methanol-isopropanol (1:1) was chosen because employing any of the other five co-solvents offered no benefit. The addition of the weak acid reduces the analytes' retention time during the mobile phase co-solvent B optimization process, but it has no discernible impact on the separation or peak shape of the steroid analytes. By choosing a 150-mm column length, the resolutions of the isomeric and isobaric analyte pairings were improved. Additional studies were conducted to optimise the flow rate, column temperature, back pressure, and make-up solvent conditions. The experimental part includes a description of the optimised separation conditions (see the method section).

This study followed a significantly altered version of the validation process outlined in EU Commission Decision 657/EEC. To complete the process, the linear range, accuracy, precision, limit of quantification (LOQ), and recovery were determined. For all validation analyses, new standard solutions of the steroid analytes were employed. Due to the existence of several interfering components, matrix-specific validation is frequently sought in steroid analysis. Plasma cannot be used as a pure, steroid-free sample due to the unknown levels of endogenous steroids present. As a result, a variety of methods, including the use of artificial plasma, surrogate analytes, standard addition, background subtraction, etc., have been used to tackle this problem.

However, in our investigation, the calibration curves for each analyte in steroid-free plasma made by charcoal stripping were used to determine the method's linear range (see the method section). For all the steroids, the r^2 square correlation coefficient was more than 0.998. It demonstrates that each analyte's signal was linear within the chosen concentration. The linearity range found in this investigation was comparable to those from SFC-MS and MS that have recently been published. The calibration samples back-calculated concentration was within 12% of the nominal value. Both significant endogenous matrix interferences and co-eluting substances were not found in the plasma samples. After injecting the highest quality solution, carry-over did not cause any issues because all analytes were undetectable from blank injections.

By contrasting the peak areas of the analytes in the reconstitution solvent with the peak areas following the extraction of steroids from plasma, the quantitative recovery of steroids in plasma was assessed. At each of the three distinct concentrations, multiple aliquots (n = 6) were evaluated. Between 82 to 107% of steroids and related deuterated internal standards were recovered on average. The signal enhancement issue was taken into consideration since we employed comparable deuterated internal standards and matrix-matched calibration in plasma.

Data from repeated analyses (n = 6) of spiked plasma samples at three distinct doses were used to determine precision and accuracy. For the majority of the steroids, the intraday and interday precisions ranged between 1% and 10%, and the accuracy was within 15%. With some exceptions, the lowest concentration values that could be calculated with a bias and a CV% lower than 15% were judged to be LOQ and were discovered to be less than 0.1 ng/mL for the majority of the steroids.

For the diagnostic assessment of steroidomics in plasma, we have been able to use the developed method in the KARMA study (Karolinska Mammography Project for Risk Prediction of Breast Cancer, KARMA), conducted at the Karolinska Institute in Sweden, one of the best-characterised breast cancer cohorts in the world. Proteomic profiling has already been used to validate the KARMA plasma samples' quality. All blood samples were processed in the high-throughput biobank at Karolinska Institute under the rigorous guidelines of a 30-hour cold-chain methodology. At study enrollment, participants completed a questionnaire to provide information on risk factors and exposures. Each trial participant approved the linkage to the national breast cancer registration and signed an informed consent form. The Stockholm ethical review board gave the study its blessing (2010/958-31). All experiments were conducted in conformity with the necessary legislation and guidelines.

In plasma from more than 700 breast cancer patients and 1400 matched controls, we analysed all 19 distinct steroids in the four major classes (oestrogens, androgens, progestogens, and corticosteroids) of steroids. Per sample, the analysis took 5.0 minutes. Separate reports will be made of the outcomes. Additionally, we have already published a few other applications based on this technique in peer-reviewed journals, demonstrating its viability for use with biological samples.

III. Concluding Remarks

The purpose of this work was to demonstrate the utility of UPSFC-MS/MS as a method for the separation and quantification of endogenous steroids in human plasma as an alternative to LC-MS/MS and GC-MS/MS. Although it is still debatable whether UPSFC-MS/MS genuinely qualifies as a "green technology" in terms of the amount of organic solvent it uses, it clearly outperforms LC-MS/MS and GC-MS/MS in terms of resolution and sensitivity. This new UPSFC-MS/MS technique allows for the simultaneous 5-minute study of 19 endogenous steroids from all four major classes. Incorporating a derivatization step before analysis increased detection sensitivity and overcame the disadvantage of longer sample preparation times. The validation data shows that these steroid analytes can be detected and quantified even in small plasma sample volumes. This method may be particularly useful for the detection of steroids in biobanked samples, where sample availability is typically constrained, in addition to research applications and routine clinical screening. Due to its high throughput and sensitivity compared to immunoassays, the new UPSFC-MS/MS approach may therefore be the method of choice for the diagnosis and monitoring of endocrine illnesses.

IV. Methods/Materials

Estrone (E1), dehydroepiandrosterone (DHEA), androsterone (AN), etiocholanolone (ECN), testosterone (T), dihydrotestosterone (DHT), androstenedione (AE), pregnenolone (Preg), 17-hydroxypregnenolone (17OHPreg), progesterone (P), 17-

hydroxyprogesterone (17OHP), pregnanol Steraloids Inc. provided the following compounds: 2,4,16,16-d4-estrone (d4-E1), 16,16-d2-androsterone (d2-AN), 2,2,4,6,6,17,21,21,21-d9-progesterone (d9-P), 2,2,4,6,6,21,21,21-d8-17-hydroxyprogesterone (d8-17-OHP), and 9,11,12,12-d4-cortisol (d, Newport, RI, USA).

Except as otherwise noted, the purest solvents and chemicals, including methylamine hydrochloride, 2,3,4-13C3-testosterone (13C3-T), 20,21-13C2-16,16-d2-pregnenolone (13C2-d2-Preg), 2,2,3,4,4-d5-allopregnanolone (d5-Allo), and 9,11,12,12-d4-corticosterone (d4-B), were obtained with the use of a Millipore (Millipore, Bedford, MA, USA). Blood donors who were in good health sent samples of human cohort plasma to the Academic Hospital in Uppsala, Sweden. Each participant's blood was drawn into EDTA vacutainer tubes by venepuncture. The plasma was separated from the blood and kept at 80 °C until further usage after centrifuging it at 3500 g for 15 minutes. Since the plasma steroid levels are remarkably consistent over a year, specific measures to preserve the plasma were not necessary.

V. Method Validation

Human plasma samples devoid of analytes were used for technique validation following method optimization. All nineteen steroids' linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and recovery were identified.

Each analyte was present at seven distinct concentrations (n = 4) on the calibration curves. For groups I and II, the spiked steroid mix solutions had concentrations between 0.05 and 30 ng/mL and 0.05 and 500 ng/mL, respectively. These parameters were chosen after taking into account the steroid concentrations in typical plasma samples that were clinically relevant. Plasma free of steroids and unspiked was chosen as the blank. 50 L of the IS mixture were added to each sample. IS was not added to the two-fold blank plasma sample during preparation. The response, which was displayed against the corresponding concentrations, was calculated by multiplying the IS concentration by the peak area ratio (analyte/IS) for each steroid. Using linear regression, the linearity was assessed.

Bias (the actual concentration subtracted from the measured concentration, expressed as a percentage of the actual concentration) and the coefficient of variation (CV%), respectively, were used to compute accuracy and precision. Six samples spiked with three different concentrations of group I analytes (low, 0.25 ng/mL; medium, 2.5 ng/mL; high, 20 ng/mL) and group II analytes (low, 2.5 ng/mL; medium, 20 ng/mL; high, 250 ng/mL) on the same day were analysed to determine intraday accuracy and precision. The triplicate analysis of spiked samples at the above doses over five consecutive days was used to calculate the interday accuracy and precision. The calibration curve was used to determine the concentration in each sample. The permissible ranges for precision and accuracy shouldn't be higher than 20%.

LOD and LOQ were established as the lowest concentrations that, by repeated injection (n = 6), generated a signal-to-noise ratio (S/N) greater than 3 and 10, respectively, with an RSD of replicates below 15%.

Absolute recovery was determined by comparing the response of spiked analytes at three different levels (low, medium, and high) in steroid-free plasma with the response of the IS concentration times the peak area ratio (analyte/IS) of each steroid obtained after replicate analysis of standard solutions (n = 6) in solvent. The peak area response of each steroid and IS from the post-extraction spiked plasma was compared to the peak area response of the standard analyte solution at the same concentration (n = 6) in order to determine the matrix effect. The ionisation behaviour of the analytes is indicated by the percentage area difference. If the observed value is larger or smaller than 100%, ionisation is either enhanced or suppressed.

Extraction

Liquid-liquid extraction is the first step in sample preparation (LLE). The process of steroid extraction was slightly altered28. In a nutshell, 50 L of plasma were combined with the IS mixture. In 2 mL of tert-butyl methyl ether, plasma steroids were extracted (MTBE). Samples were gently vortexed for 10 minutes, followed by 10 minutes of centrifugation at 1000 g. The solvent was evaporated under a stream of nitrogen gas after the supernatant was collected. By adding 0.05 mg/mL BHT to the extraction solvent, the steroids were shielded from oxidation during the extraction process (MTBE). The most typical solvents used for LLE7 are MTBE, diethyl ether, dichloromethane, and a combination of hexane and diethyl ether. It was discovered that MTBE works best for an acceptable..

Derivatization

The derivatization reagent was methylamine hydrochloride (20 mg/mL) in anhydrous pyridine. The samples were incubated at 60 °C for 45 min after the addition of 100 L of reagent. In 50 L of 0.1% formic acid in methanol-isopropanol, the oxime derivatives were dissolved after the surplus reagent was evaporated under a stream of nitrogen (1:1). Prior to UPSFC-MS/MS analysis, samples were maintained at or below 20 °C, as detailed below.

VI. Determination of Steroid Concentrations by UPSFC-MS/MS

The analysis was done using a Xevo TQ-S triple quadrupole mass spectrometer connected to an Acquity UPC2 (Waters Corporation, Milford, USA) system for ultra-performance supercritical fluid chromatography-tandem mass spectrometry (UPSFC-MS/MS) (Waters, Milford, USA). A column oven, an autosampler, a binary solvent supply pump, and a back pressure regulator were included in the UPSFC system. A commercial interface kit (Waters) consisting of two T-pieces that allowed for backpressure control and post-column infusion with a make-up solvent was used to link UPSFC to the mass spectrometer.

Three distinct stationary phases (Acquity UPC2 columns (Waters, Milford, USA), BEH, BEH 2-EP, and CSH fluoro-phenyl (3.0 mm, 100 mm, 1.7 m)) were used to measure the column selectivity. An Acquity UPC2 BEH column was used to separate the nineteen steroids. With the mobile phase flowing at a rate of 2 mL/min, the column was maintained at 40 °C. The gradient programme began with 98% A (CO2) and 2% B (0.1% formic acid in methanol-isopropanol (1:1)), which were maintained for 0.1 min. From there, the gradient linearly climbed to 17% B over 3 min, was kept at 17% B for 0.5 min, and then decreased to 2% B over 0.5 min The ionic liquids were then eluted from the instrument for 1 min at 2% B, bringing the total separation duration to 5 min. The injection volume was 1.0 L, and the back pressure was predetermined to be 1500 psi (103.4 bar). A make-up solvent (0.1

percent formic acid in methanol) was used to help elute the sample from the SFC system into the MS system at a flow rate of 0.2 mL/min.

Electrospray ionisation in the positive ionisation mode (ESI+) was used for mass spectrometric detection, with capillary voltages of 2.8 kV, cone voltages of 30 V, and source offsets of 30 V. The collision gas was argon (0.15 mL/min), and the desolvation gas was nitrogen. A source temperature of 150 °C and a desolvation temperature of 500 °C were both maintained. Desolvation gas flow and cone gas flow were kept at respective rates of 750 L/h and 150 L/h. The gas flow on the nebulizer was set at 101.5 psi (7.0 bar). To maximise product ion production, collision energy was changed.

The m/z 100–600 range was chosen as the data gathering range. Using IntelliStartTM in infusion mode, standard solutions of the steroids at a concentration of 10 g/mL were infused into the source at a rate of 10 l/min. Each analyte's mass spectra were captured in MS and MS/MS modes. The collision energy and scan dwell duration were established in accordance with Table 1 for the quantification, which used the multiple reaction monitoring (MRM) approach. By analysing each of the standard steroids individually (50 ng/mL), the MS/MS settings and the procedure were verified. Software called MassLynx TM4.1 was used to gather, analyse, and process the data (Waters, Milford, USA). Utilising the appropriate internal standard, steroids were quantified.

VII. CONCLUSION:

These high-tech techniques are incredibly valuable for acquiring structural data on specific steroids and their metabolites. A variety of chemical derivatization techniques are frequently used in conjunction with GC-MS analysis of steroids and their metabolites in biological samples.

The majority of methods for steroid analysis that have been disclosed are concentrated on identifying a small number of steroids within one or two classes.

Before analysis, these steroids underwent dansyl chloride derivatization.

REFERENCES:

1. Gaikwad, N. W. Ultra performance liquid chromatography-tandem mass spectrometry method for profiling of steroid metabolome in human tissue. *Anal. Chem.* **85**, 4951–4960 (2013).
2. Lusa, S., Heino, S. & Ikonen, E. Differential mobilization of newly synthesized cholesterol and biosynthetic sterol precursors from cells. *J. Biol. Chem.* **278**, 19844–19851 (2003).
3. Payne, A. H. & Hales, D. B. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr. Rev.* **25**, 947–970 (2004).
4. Kushnir, M. M., Rockwood, A. L. & Bergquist, J. Liquid chromatography-tandem mass spectrometry applications in endocrinology. *Mass Spectrom. Rev.* **29**, 480–502 (2010).
5. Krone, N. *et al*. Gas chromatography/mass spectrometry (GC/MS) remains a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry (LC/MS/MS). *J. Steroid Biochem. Mol. Biol.* **121**, 496–504 (2010).
6. Kushnir, M. M. *et al*. Liquid chromatography tandem mass spectrometry for analysis of steroids in clinical laboratories. *Clin. Biochem.* **44**, 77–88 (2011).
7. Abdel-Khalik, J., Björklund, E. & Hansen, M. Simultaneous determination of endogenous steroid hormones in human and animal plasma and serum by liquid or gas chromatography coupled to tandem mass spectrometry. *J. Chromatogr. B* **928**, 58–77 (2013).
8. Gouveia, M. J. *et al*. Mass spectrometry techniques in the survey of steroid metabolites as potential disease biomarkers: A review. *Metabolism* **62**, 1206–1217 (2013).
9. Schumacher, M. *et al*. Analytical challenges for measuring steroid responses to stress, neurodegeneration and injury in the central nervous system. *Steroids* **103**, 42–57 (2015).
10. Jeanneret, F. *et al*. Evaluation of steroidomics by liquid chromatography hyphenated to mass spectrometry as a powerful analytical strategy for measuring human steroid perturbations. *J. Chromatogr. A* **1430**, 97–112 (2016).
11. Marcos, J. & Pozo, O. J. Derivatization of steroids in biological samples for GC-MS and LC-MS analyses. *Bioanalysis* **7**, 2515–2536 (2015).