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From a single steroid to the steroidome: Trends and analytical challenges

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ABSTRACT

For several decades now, the analysis of steroids has been a key tool in the diagnosis and monitoring of numerous endocrine pathologies. Thus, the available methods used to analyze steroids in biological samples have dramatically evolved over time following the rapid pace of technology and scientific knowledge. This review aims to synthetize the advances in steroids' analysis, from classical approaches considering only a few steroids or a limited number of steroid ratios, up to the new steroid profiling strategies (steroidomics) monitoring large sets of steroids in biological matrices. In this context, the use of liquid chromatography coupled to mass spectrometry has emerged as the technique of choice for the simultaneous determination of a high number of steroids, including phase II metabolites, due to its sensitivity and robustness. However, the large dynamic range to be covered, the low natural abundance of some key steroids, the selectivity of the analytical methods, the extraction protocols, and the steroid ionization remain some of the current challenges in steroid analysis. This review provides an overview of the different analytical workflows available depending on the number of steroids under study. Special emphasis is given to sample treatment, acquisition strategy, data processing, steroid identification and quantification using LC–MS approaches. This work also outlines how the availability of steroid standards, the need for complementary analytical strategies and the improvement of calibration approaches are crucial for achieving complete steroidome quantification.

1. Introduction

Steroids are a group of lipids with a common cyclopentanoperhydrophenanthrene skeleton that play a critical role in the organism at endocrine, paracrine and intracrine regulation levels [1]. Corticosteroids (glucocorticoids and mineralocorticoids) are involved in several biological processes, including stress response and immune response, and regulation of inflammation and carbohydrate metabolism. Sex steroids (such as progestogens, androgens and estrogens) mediate reproductive functions and the development of sexual characteristics, among other functions [2–4]. Steroids are largely metabolized through phase I and II reactions to modulate their receptor-binding capacity and, thus, their biological activity, and to increase their water solubility for urinary and bile excretion [2]. In phase I metabolism, the steroid structure itself will undergo chemical transformations such as oxidation and reduction, while phase II metabolism mainly consists of esterification reactions or conjugations between the steroids and highly polar, charged groups, commonly sulfate and glucuronate [5]. Steroid biochemical pathways comprise synthesis (involving mainly cytochromes P450 (CYPs) and hydroxysteroid dehydrogenases (HSDs)), peripheral metabolism (active/inactive forms; mediated by HSDs, steroid-5α-reductase (SRD5), aldo-keto reductases (AKRs) or CYPs) and hepatic metabolism prior to excretion (through CYPs and AKRs) [2]. A schematic representation of steroidogenesis with the major circulating hormones and their main unconjugated metabolites is presented in Fig. 1. Since the 1960s, the analysis of selected hormones of clinical relevance has been routinely performed by gas chromatography (GC) coupled to mass spectrometry (MS), requiring sample preparation steps such as derivatization or hydrolysis reactions [6]. In the 1980s, the first commercial kits for steroid measurements using automated immunoassay instruments became available. As the cost of such analyses was relatively low and the technique was easy to perform, immunoassays rapidly gained a place in clinical laboratories for steroid analysis coexisting with GC—MS [7]. However, immunoassays also showed problems

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regarding sensitivity, selectivity, and lack of reproducibility [8–10], what accelerated the emergence of liquid chromatography (LC) coupled to MS for steroid analysis. For many years, the complementary use of chromatography techniques (GC and LC) coupled to MS has become very popular for steroid analysis due to their robustness, sensitivity, selectivity and capacity to monitor different analytes at the same time [11]. In addition, LC—MS permitted the direct detection of steroids and steroid conjugates (i.e., phase II metabolism) without the need for derivatization [12]. The interested reader is referred to any of the existing excellent reviews on the topic for an in-depth view of the different aspects in the field [1,7,11,13,14].

For several decades, alterations in the steroid metabolome have been largely investigated for steroid-related endocrine diseases [2]. Traditionally, clinicians have evaluated specific diagnostic steroid ratios based on biochemical pathway knowledge about the precursor/product relationships, thus targeting individual enzymes typically found in steroidopathies, such as congenital adrenal hyperplasia (CAH) or Cushing's disease [15]. However, there has been a growing interest in expanding this limited approach to a larger and more representative group of steroids in order to have a better understanding of the mechanisms behind endocrine pathologies with the goal to develop better diagnostic strategies.

Overall, the simultaneous quantification of an increasing number of steroids has become a common strategy in clinical and research laboratories [2]. Recent advances in steroid analyses (with an emphasis on MS technology and methodologies) combined with computational approaches have facilitated multi-steroid profiling in clinical practice [16,17]. The present review aims to synthetize the advances on the steroids' quantification strategies with a focus on LC–MS, starting from the ones limited to few steroids, up to the comprehensive monitoring of the steroidome.

2. Challenges in steroids analysis by LC–MS

The simultaneous determination of a large panel of steroids (precursors, hormones and their principal metabolites) has become an attractive approach to study pathological conditions linked to hormonal imbalances. However, several challenges must be overcome during steroid analysis with LC–MS based techniques. The main issues for analyzing the steroid profile in biological matrices include i) their large dynamic range, ii) their extraction from complex samples, iii) the selectivity of the analytical techniques (chromatographic separation and mass spectrometry), and iv) the ionization efficiency on a particular subfamily of steroid compounds (i.e., estrogens). A schematic overview of the challenges found in steroid analysis is summarized in Fig. 2.

2.1. Dynamic range

A major constraint in steroid analysis is the large range of concentrations at which they can be found in a particular matrix and also the remarkable differences between different biological compartments or fluids. As an example, in circulating serum, the steroid concentrations typically range from 10−0.1 μmol/L for DHEAS or cortisol to $<10^{-4}$ μmol/L for estrogens such as 17β-estradiol or estrone (in males or postmenopausal females)[2]. However, in human urine, the steroid abundances span from 10 to 10^2 μmol/mmol creatinine for the main metabolites, whereas they are in the 10^{-4} to 10^{-1} μmol/mmol creatinine range for precursor hormones and minor metabolites [18,19]. Fig. 3 (shows the analysis of 32 steroids (androgens, mineralocorticoids, glucocorticoids and precursors) in 24 h urine, evidencing the remarkable variations found across the individuals and the molecules [20]. This is also the case in less common matrices. For example, endogenous cortisol in hair has been detected in a large concentration range (from approx. 1 pg/mg to 100 pg/mg) and such concentrations are correlated to its endogenous time-dependent release in response to stress, or in different pathological conditions [21,22]. Seminal fluid presents a rather low steroid abundance (i.e., testosterone, androstenedione or cortisol) compared to plasma or urine [23]. Indeed, the range of the reported seminal steroid concentrations is very broad, especially in the case of key steroids such as testosterone, what might be due to the selected methodology used rather than actual differences [24]. Furthermore, some steroids show a remarkable physiological variability in the population what yields broad reference ranges that make it difficult to classify a certain individual as a healthy or a pathological one [19].

2.2. Steroid extraction

Another relevant limitation in steroid analysis is the efficient extraction of analytes exhibiting diverse polarities from different matrices [25]. Steroid extraction mainly depends on i) the nature of the matrix, ii) the cleaning and pre-concentration steps and iii) the strategies to detect steroid conjugates. Taking into account such factors, steroid extraction efficiency can be notably increased for optimal measurement.

2.2.1. Nature of the matrix

Steroids must be extracted from samples before they can be analysed,

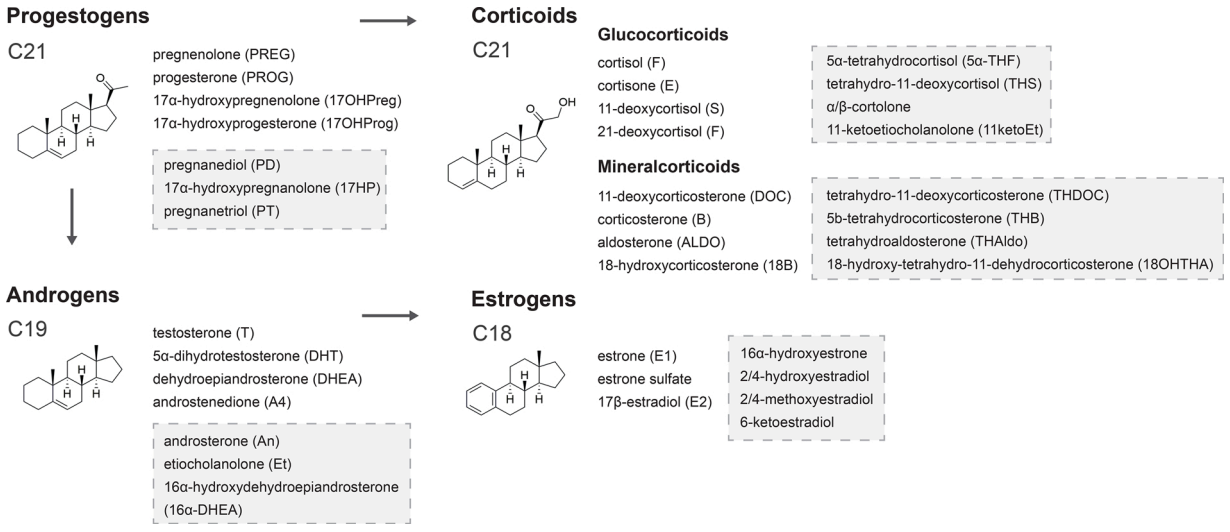

Fig. 1. Scheme showing the main circulating steroid hormones (progestogens, corticoids, androgens and estrogens) and their principal unconjugated metabolites excreted in urine (in grey boxes).

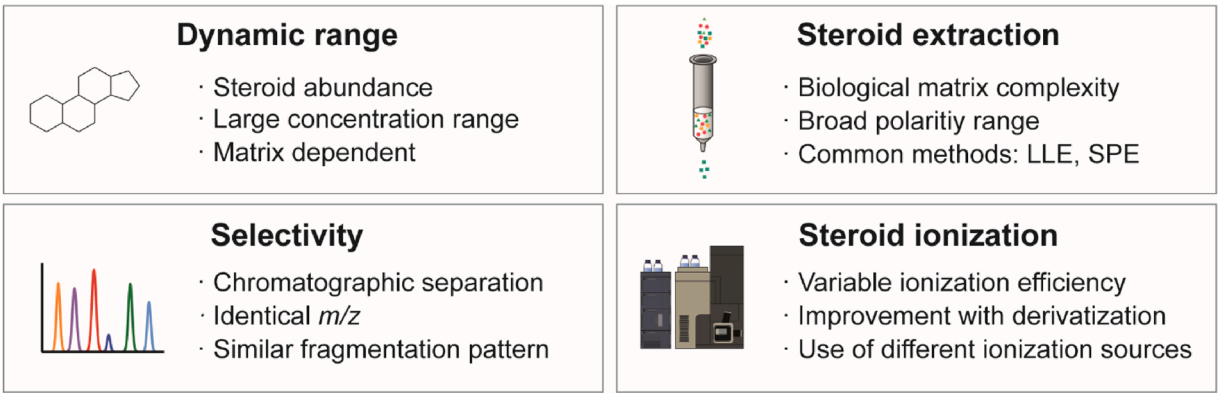

Fig. 2. Overview of the main challenges of the steroid analysis with chromatography techniques coupled to mass spectrometry.

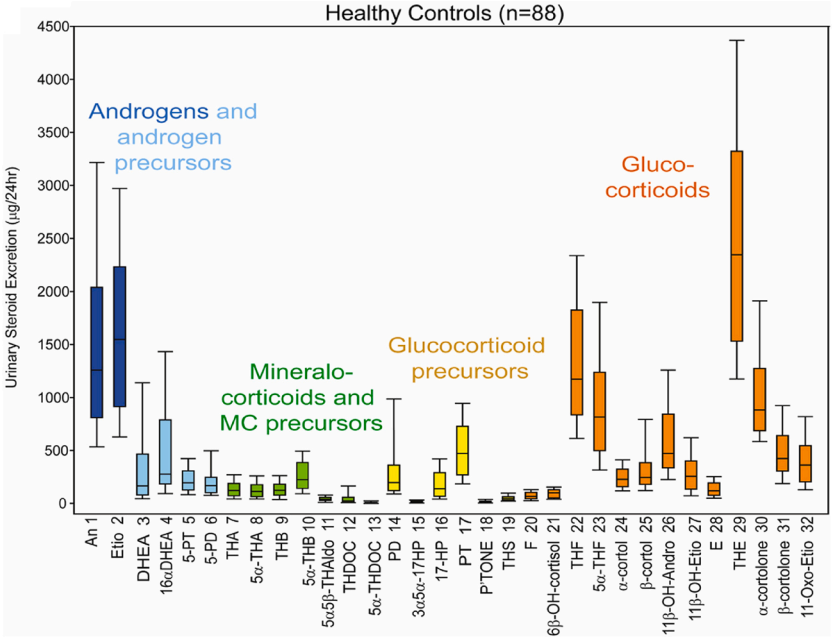

Fig. 3. The 24-h urinary steroid metabolite excretion in healthy controls (n = 88). Box plots represent median and interquartile ranges; the whiskers represent 5th and 95th percentile, respectively. Figure adapted from Arlt W et al. [20].

and specific methodologies have to be designed to cope with different matrices. Protein-rich matrices such as plasma [26], amniotic fluid [27] or seminal fluid [23,24], usually require a protein removal step (i.e., protein precipitation), whereas this step can be avoided in matrices such as urine [18,28], saliva [29] or breast milk [27]. Solid matrices, including hair [21,22,30], nails [31] or tissues [32], require milling and homogenization. Another key aspect of steroid measurement in biological matrices is the amount of sample available for the analysis. Large volumes of urine [18] (10 mL to several litres, depending on the collection period) are commonly accessible in contrast to other matrices such as blood plasma (from 50 µL in rodents, to 5 mL or more in mammals), amniotic fluid (100 µL to hundreds of mL) [27] or cerebrospinal fluid (1−5 mL) [33]. Restricted-volume samples will be more challenging when targeting low-concentration compounds, since a pre-concentration step is not feasible.

2.2.2. *Cleaning and pre-concentration steps*

These steps serve to remove interferences and increase the concentration of the steroids injected into the instrument [34–36]. As previously stated, steroids have diverse physicochemical properties, including a wide range of polarities (i.e., the LogP for cortisol is 1.61, while that for estradiol is 4.01 [37]), what hinders their simultaneous extraction from a biological sample. During metabolism, steroids generally become more hydrophilic by hydroxylation reactions or conjugation of glucuronic or sulfuric acids [38]. Currently, the most common extraction method for low-polarity (or non-polar) steroids (such as androgens, estrogens and progestogens) are LLE or its supported alternative, SLE. Their relatively high affinity towards organic low-polarity solvents allows to remove polar interferences [39,40]. Historically, dichloromethane has been widely used while, more recently, tert-butyl-methyl ether is gaining popularity due to its better safety profile. In the case of steroids with hydroxyl groups, such as glucocorticoids and mineralocorticoids, they can be extracted with more polar organic solvents, such as ethyl acetate [28]. For direct measurement of phase II metabolites (steroids' mono- or bis-conjugates forming glucuronides, sulfates, cysteinyls, or combinations thereof), SPE is routinely used for extraction prior to LC—MS analysis [5,12,41–44]. Polymeric SPE cartridges with balanced lipophilic and hydrophilic properties make possible a better retention and isolation of such compounds [23,45–47].

2.2.3. Conjugate hydrolysis

The scarcity of conjugated steroid standards prevents the preparation of calibration curves with pure reference standards, making the hydrolysis of the samples prior to their LC–MS analysis a convenient step in sample preparation for conjugates' analyses (indirect measurement) [48]. Deglucuronidation and desulfation are commonly performed with enzymes such as β-glucuronidase (i.e., that from *Escherichia coli*) [49] or sulfatase (i.e., that from *Helix pomatia*) [50]. Despite the large use of this indirect measurement of phase II steroids, a relevant drawback is the reproducibility and overall reliability of a complete deconjugation step. The efficiency of the β-glucuronidase enzyme depends on the structure of the glucuronide, and it has been reported that for some steroids, hydrolysis may be incomplete due to the presence of enzyme inhibitors in the urine [49]. Indeed, some conjugates (such as 3α-glucuronide-6β-hydroxyetiocholanolone, a marker of testosterone oral administration) have been shown to be resistant to enzymatic hydrolysis [51,52]. Furthermore, when dealing with bi-conjugates, the complexity of the presence of 2 conjugates challenges the efficiency of the enzymatic activity and in some cases hydrolysis may not be complete [49]. Importantly, the efficient deglucuronidation and desulfation of steroids in urine samples still remains a constraint to achieve controlled and reproducible sample preparation protocols.

2.3. Selectivity

Another key aspect of steroid analysis is compound selectivity. Steroids are known to have very similar structures (overlapping chromatographic retention times, masses, and fragmentation patterns) rendering crucial the selectivity of the applied GC–MS and LC–MS methods.

2.3.1. Chromatographic separation

As briefly mentioned in the Introduction section, GC has been used since the 1960s due to its high steroid separation performance. A derivatization step (using for example trimethylsilylation) provides compound stability at high temperatures and turns the analytes more hydrophobic and volatile allowing their separation in the GC column [15,53]. In the case of LC, non-conjugated steroids usually exhibit good chromatographic behaviour with C18 columns due to their lipophilicity [45,48], allowing for high peak capacity, good separation efficiency, sharp peak shapes and robust annotation of steroids based on their repeatable retention times. As an example, Fig. 4 shows the chromatographic separation of 28 steroids in a human sample of seminal fluid [23] using LC–MS. The predictable chromatographic behaviour of steroids, makes possible to develop models that allow for prediction of their retention times based on some molecular descriptors with good accuracy and precision [54,55]. However, the existence of numerous isomers and coeluting species having the same lipophilicity in C18 columns challenges chromatographic separation either in an experimental or in a predictive approach. This fact has led to the use of new technologies such as core-shell particles to improve the separation performance [56], and to the search for alternative stationary phases such as biphenyl [57] or pentafluorophenyl [58] chemistries, showing complementary selectivity to C18 through additional types of interactions with the steroid molecules. The use of other chromatographic strategies, such as supercritical fluid (or convergence) chromatography, has also shown a promising performance compared to LC or GC in clinical and doping analyses [59–62].

2.3.2. Mass spectrometry

Because of their shared backbone and the existence of a rich variety of stereoisomers, many steroids present an identical chemical formula and show similar fragmentation patterns, which complicates their univocal annotation using either HRMS or MS/MS approaches. Fragments from steroids can be obtained and compared with standard references. The collision-induced dissociation mechanism typically found in LC–MS settings with electrospray ionization (ESI) sources yields less reproducible fragmentation patterns than with electronic impact ionization in GC–MS. However, the use of high-resolution mass spectrometers (such as quadrupole time-of-flight, Orbitrap and Fourier transform ion cyclotron resonance (FT-ICR) instruments) can help elucidate the molecular formula of steroids having the same nominal mass. For example, 6-ketoestriol ($C_{18}H_{22}O_4$, nominal mass 302.4 Da and accurate mass $[M+H]^+$ 303.15909 Da) can be distinguished from 11β-hydroxyandrostenedione ($C_{19}H_{26}O_3$; nominal mass 302.4 Da and $[M+H]^+$ 303.19547 Da) much more efficiently using HRMS than low-resolution mass spectrometers [63–65]. Recently, many MS manufacturers have started selling instruments capable of measuring the collisional cross-sections (CCS) of the ions by means of the so-called ion-mobility MS (IMS). Unfortunately, this parameter has been shown to have a high degree of correlation with the mass of steroid ions, thus affording little or no orthogonal information allowing to improve the annotation and identification of these molecules except in cases where different adducts or flexible phase-II structures have to be characterized [66,67].

2.4. Steroid ionization

The presence of ionizable functions in a number of steroids facilitates their ionization and detection using ESI sources. However, for low-concentration steroids, an excellent ionization efficiency is needed in order to guarantee a suitable sensitivity of the technique. In such cases, ionization has to be improved by means of different resources [48]. The use of derivatization agents such as picolinic acid for the hydroxyl groups, and hydroxylamine or methyloxime for the derivatization of carbonyls [48], is recommended for increasing ion formation in LC-ESI-MS. However, the reagent used depends on i) the structural characteristics of the selected steroids ii) the biological matrix and iii) the equipment used. As a consequence, it increases the complexity of the

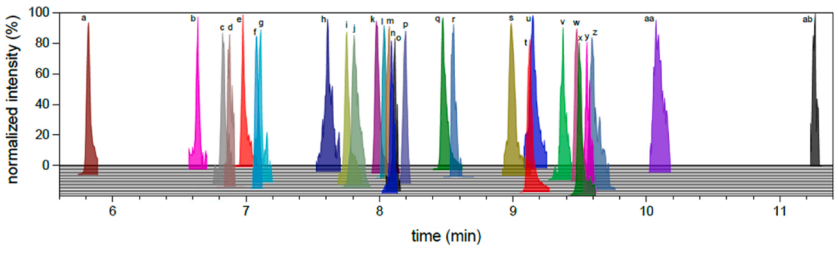

Fig. 4. Chromatogram of a 28 steroids profile found in a sample of human seminal fluid and separated on a C18 column. From left to right, peaks correspond to: (a) 2-hydroxyestriol, (b) 16a,17b-estriol, (c) 5a-androstane-3b,7a,16b-triol, (d) 6b-hydroxytestosterone, (e) cortisol, (f) cortisone, (g) 19-hydroxyandrostenedione, (h) 3a,5b-tetrahydrocortisone, (i) 11-dehydrocorticosterone, (j) androst-5-ene-3b,16a,17a-triol, (k) glycocholic acid, (l) 11b-hydroxyandrostenedione, (m) 11-deoxycortisol, (n) glycoursodeoxycholic acid, (o) etiocholanolone-3a-glucuronide, (p) 3a,21-dihydroxy-5a-pregnan*1120-dione, (q) 11-ketoetiocholanolone, (r) 11a-hydroxyprogesterone, (s) cholic acid, (t) glycochenodeoxycholic acid, (u) testosterone, (v) 17a-hydroxypregnenolone, (w) androstane-3b,5a,6b-triol, (x) androstenedione, (y) DHEA, (z) 17a-hydroxyprogesterone, (aa) 5a/b-dihydrotestosterone, (ab) pregnenolone. Peak heights were plotted as normalized intensity (100 %). Taken from Olesti E et al [23].

sample preparation process and the total cost. Furthermore, the derivatization reaction can produce multiple derivative products (especially when the reaction is not complete) that may interfere in steroid annotation. Still, one of the main drawbacks of steroid derivatization is the lack of a single protocol capable of covering the whole range of steroids [48,68,69].

In LC–MS, mobile phase additives are widely used to improve separation and boost ionization. The addition of formic acid is a good example for that, as it does not only reduce interactions between analytes and deprotonated silanols in reversed-phase columns, but it also improves analyte ionization in ESI in positive mode. Ammonium formate [28] or ammonium fluoride [70] can also improve steroids' ESI ionization by facilitating the production of adducts. However, the formation of adducts strongly depends on each steroid structure and on the mobile phase composition, and it may affect the sensitivity and specificity of these ions and the interferences produced by the matrix [71].

Using ESI polarity switching (i.e. quickly alternating negative and positive ionization in the same analytical run) can increase throughput by combining the detection of positively and negatively ionizable steroids in the same LC–MS run. Nevertheless, it will be done at the expenses of acquiring half the data points per peak, what can compromise the quality of peak integration in fast quantitative applications. Another drawback is that LC additives will typically improve only one of the two ionization modes, thus rendering the other less efficient, contrary to independently tuning two separate LC–MS experiments by adding specific additives for each ESI mode [72].

Another strategy for improving the detection and ionization of steroids is the use of alternative ionization modes. One of such possibilities is atmospheric pressure chemical ionization (APCI), which can provide more sensitive detection of some steroid families, such as estrogens [73]. Likewise, the use of atmospheric pressure photoionization (APPI) can reduce background interferences for improving some steroid signals (i.e., androgens or estrogens) [74].

3. Steroid quantification

Despite the discussed challenging issues found in steroid analysis, the quantification of a limited set of steroids is routinely performed at many clinical and anti-doping laboratories, metabolomics platforms, industries and other research facilities. Steroid quantification is crucial for a better understanding, diagnosis, prognosis and treatment of many endocrine pathologies and has acquired major relevance in competitive sports.

In this section, we review the different strategies for steroid quantification according to the number of compounds to be tackled. While the quantification of a low number of steroids (1–15) is routinely performed, an extensive characterization of the whole steroidome (above 200 steroids) is ideal but difficult to achieve. Nevertheless, it is only through such measurements that access to the complete panel of enzymes involved in steroid synthesis and metabolization could be achieved. In Table 1, we present a summarized overview of the different strategies (from the study design, sample treatment, analytical strategies, and data treatment to its applications) for steroid analysis for a selected set of clinical hormones, a restricted panel of steroids and the steroidome.

3.1. Analysis of a selected set of clinical hormones (1–15 steroids)

The measurement of a single steroid hormone in plasma is the gold-standard bioanalytical test for numerous endocrine pathologies. Circulating testosterone is quantified for the diagnosis of hypogonadism, chronic heart failure and testicular dysfunction in men and for the diagnosis and treatment of polycystic ovary syndrome, alopecia, acne and hirsutism cases in women [10,75–77]. In the same spirit, cortisol is widely monitored for hypo- and hyper-adrenal disorders (such as adrenocortical carcinoma, Cushing's disease and congenial adrenal hyperplasia among other pathologies) [17,78–80], progesterone monitored for ovulation status [81,82] and aldosterone for primary aldosterone disorders (commonly caused by an excess production of aldosterone) [83,84]. Numerous analytical methodologies have been developed for the measurement of a previously selected set of steroids using LC—MS, from strategies to quantify a single hormone (i.e., aldosterone [85,86], cortisol [87], estradiol-17β [88] or testosterone [89,90]) to the analysis of a small group of hormones that are generally relevant for clinical purposes [91–94].

Moreover, some commercial kits are available and routinely used in laboratories (i.e., CHS™ MSMS Steroids Tool Box from Perkin-Elmer [95] and/or the SteroIDQ® Kit from Biocrates [96]). Such kits usually facilitate most steps of the analytical workflow, from sample collection and preparation, to derivatization, separation, detection and even data treatment. Alternatively, in-house methodologies can be developed [28]. Although significant advances are being done in untargeted acquisition for quantitative analyses [97] targeted acquisition is virtually the only option when it comes to quantification due to its better performance in terms of selectivity and sensitivity. With the goal to

Table 1
Main characteristics of the different strategies for the analysis of a selected set of clinical hormones, a panel of steroids and the steroidome.

Steroid analysis		Selected set of clinical hormones	Selected panel of steroids	Steroidome
Study design	Nº of steroids	1 to 15	15 to 50	50 to 200
	Type of steroids	Clinically relevant hormones	Clinical hormones and metabolites	Steroidome
	Coverage	Selected set of steroids	Selected set of steroids (commonly families of steroids)	Maximum metabolite coverage
	Hypothesis	Closed	Closed	Open
Sample treatment	Sample preparation	Optimized	Broad	Generic
	Steroid extraction	Commercial kits, SPE, SLE, LLE	SPE, SLE, LLE	
Analytical Strategy	Equipment	Immunoassay, GC-MS/MS, LC-MS/MS	GC-MS/MS, LC-MS/MS GC-HRMS, LC-HRMS	
	Acquisition mode	Mainly targeted mode	Mainly targeted and few examples with untargeted mode	Mainly untargeted mode
	Quantification output	Concentration	Concentration / Relative estimation	Relative estimation
Data treatment	Data Processing	Integration peak, quantification strategy		Noise filtering, peak picking, ion annotation, alignment, normalization, identification
	Compound identification	Mainly pre-acquisition selection	Pre and post-acquisition identification	Mainly post-acquisition identification
	Software for data treatment	Vendor or open-source quantitation softwares		Dedicated metabolomics software
Application	Use	Diagnosis, disease monitoring	Diagnosis, disease monitoring, novel steroid discovery	Exploratory analysis, novel pathways
	Facilities	Clinical laboratories, research facilities and anti-doping community		Research facilities and anti-doping community

guarantee a reliable and robust measurement of the analyte over time, method validation must be carried out in accordance to international guidelines such as the ones proposed by the FDA [98] or the EMA [99]. It comprises the assessment of the figures of merit such as linear range, matrix effect, precision, trueness, selectivity, analyte stability, total analytical error (TAE) or analyte uncertainty [100].

Novel studies are currently investigating the reproducibility of validated steroid measurements (such as adrenal steroids) among different LC–MS/MS assays in different European laboratories [101]. The main preliminary result found by this ring study was that the result robustness was compound-dependent. Steroids such as cortisone or corticosterone presented good inter-laboratory reproducibility, while compounds such as cortisol or 11-deoxycortisol had poorer reproducibility. However, these results are still under evaluation, and further studies are required to identify and correct the major sources of variability [101]. In general, a thorough initial validation and a continuous monitoring of the analytical methods performance (by means of internal and external quality assessment, for instance) is much needed to achieve robust and reliable steroid concentration results in targeted methods for a limited number of steroids [102].

3.2. Analysis of a selected panel of steroid compounds (15–50 steroids)

In the past few decades there has been growing interest in quantifying a larger panel of steroids instead of monitoring single molecules. The goal was to gain extended coverage of endocrine pathologies, and this tendency has been reflected in some relevant research [103]. Many examples can be found for this sort of analyses, including validated methods for the quantification of estrogens [104,105], glucocorticoids [17], mineralocorticoids [106,107], androgens [108,109], bile acids [110], ketosteroids [111], sulfates [43,112] or even a panel of hormones and their principal phase I metabolites [28,113]. A study performed by Pozo et al. showed that there is a deep adrenal steroidogenesis imbalance (affecting 40 compounds of the biosynthesis of cortisol and their metabolites) in patients with acute intermittent porphyria [114]. In a recent review performed by Shackleton et al., the authors demonstrated the relevance of monitoring a panel of steroids for the diagnosis and management of inborn adrenal disorders [16], as well as for obtaining a differential diagnosis of adrenal incidentalomas [115]. Another example showed that different steroid profiles (n = 15 steroids) were found in subpopulations of patients with Cushing's disease, which could contribute to simplifying the diagnosis-screening test [116]. The quantification of a panel of 36 urinary steroids allowed to increase the understanding of hormone imbalance cirrhosis in patients with early hepatocellular carcinoma [117]. Another study investigated the simultaneous alterations of several steroid hormones for the diagnosis of primary liver cancer [118] and hypertension [119]. Furthermore, the quantification of an extensive panel of steroids has also been largely applied for anti-doping purposes [120]. Indeed, ten years ago the World Anti-Doping Agency (WADA) started to longitudinally measure a panel of endogenous steroids for each athlete to detect potential exogenous administration for doping purposes [121,122]. The quantification of this steroid profile (plus steroid ratios) was included in the athlete biological passport (ABP), which could be enlarged by monitoring new relevant biomarker candidates [123–125]. In summary, the quantification of a panel of selected steroids is mainly performed in clinical laboratories (where a large quantity of samples usually needs to be rapidly and robustly analysed [72]), in research facilities (typically for investigations related to diagnosis, understanding and treatment of endocrine pathologies, among other applications [11,16]) and in the anti-doping WADA-accredited laboratories (for monitoring ABPs and ensuring fair play among professional athletes [126,127]).

In the quantification of panels of between 15 and 50 steroids, the most widely used methodologies are an evolution of the ones depicted in the previous section: targeted, fully validated methods. Remarkably, since internal standards will not always be available for all the quantified steroids, herein it becomes more common the use of similar internal standards playing this role for a number of analytes. Typically, a internal standard per chemical family can be a good tradeoff, although matrix effects can be very different even for minute differences between the retention times of the analyte and the internal standard. Steroids are extracted according to their chemical properties and grouped in metabolic families to facilitate the biological interpretation. In general, these methodologies involve an optimized extraction step to clean and preconcentrate the steroids from the sample with SPE or LLE. Then, the most common acquisition mode is targeted (normally using multiple reaction monitoring mode of triple quadrupole or ion trap MS instruments). However, other studies based on a targeted strategy implement an LC-HRMS approach (i.e., parallel reaction monitoring or multiplexed ion monitoring mode with the Orbitrap) for the quantification of steroids [128–133]. The use of LC-HRMS equipment for quantification purposes has the benefit of generating additional information of the selected steroid (such as the exact mass) that can contribute to improving the selectivity of the methodology. Finally, the use of full scan modes (in HRMS) [123,124] or precursor ion scan, neutral loss scan and theoretical selected reaction monitoring strategies (in triple quadrupole or ion trap) [45,134] are typically reserved for exploratory and steroid discovery approaches rather than quantification strategies.

3.3. Analysis of the steroidome (50 to more than 200 steroids)

The steroidome encompasses the detection of the steroid metabolites (precursors, hormones, metabolites and sub-products) of a living organism, and at present, their absolute quantification remains immature. As far as we know, there are no examples of the comprehensive quantification of the steroidome in humans, despite the potential benefits of the simultaneous monitoring of all the involved molecules. In fact, the complete picture of the steroidome is expected to contain a large number of unknown compounds. New natural (and synthetic) steroids will be discovered as the advances in the instrumentation will push its current boundaries in terms of sensitivity and screening capacity. For instance, recent evidence has unveiled the presence of novel 11-oxygenated steroids showing an activity similar to those of testosterone and DHT [135]. With regard to the known steroidome, there are few studies that simultaneously monitor a wide range of steroid metabolites (approx. 100–150) of both phase I and II (mainly glucuronides and sulfates) in urine [136,137]. The goal of such studies was to enlarge the coverage of the steroidome for simultaneously monitoring a large panel of compounds and discovering novel biomarkers for diagnosing pathologies [138]. In the literature, we found examples where the steroid profile contributes to the study of diseases such as polycystic ovary syndrome [139], the evaluation of adrenal tumours [140], cardiovascular diseases [141] and other physiological and non-physiological statuses [142]. Furthermore, the steroid profile has also been studied as an approach for discovering biomarkers in the doping control field [123,124].

The current analysis of the steroidome follows a generic workflow that goes from steroid extraction from the biological sample to the relative estimation of the metabolites (schematized in Fig. 5). In order to avoid extraction bias towards certain families of steroids, a very generic workflow is usually proposed for steroidome analysis. However, some steroids require pre-concentration steps (and cleaning steps) for their detection and quantification. Therefore, a compromise between efficient steroid extraction, chromatographic separation and steroid ionization is necessary to monitor a large number of compounds. Despite being resource consuming, the combination of complementary approaches (SPE, LLE, dilute-and-shoot, etc.) [12], can facilitate the comprehensive extraction of the steroidome. Data processing depends on the acquisition strategy (targeted or untargeted approaches). In targeted strategies, fragmentation information is employed to confirm peak identity, and validated methods are used to provide quantitative results on a limited number of variables. On the other hand, untargeted steroidomics require

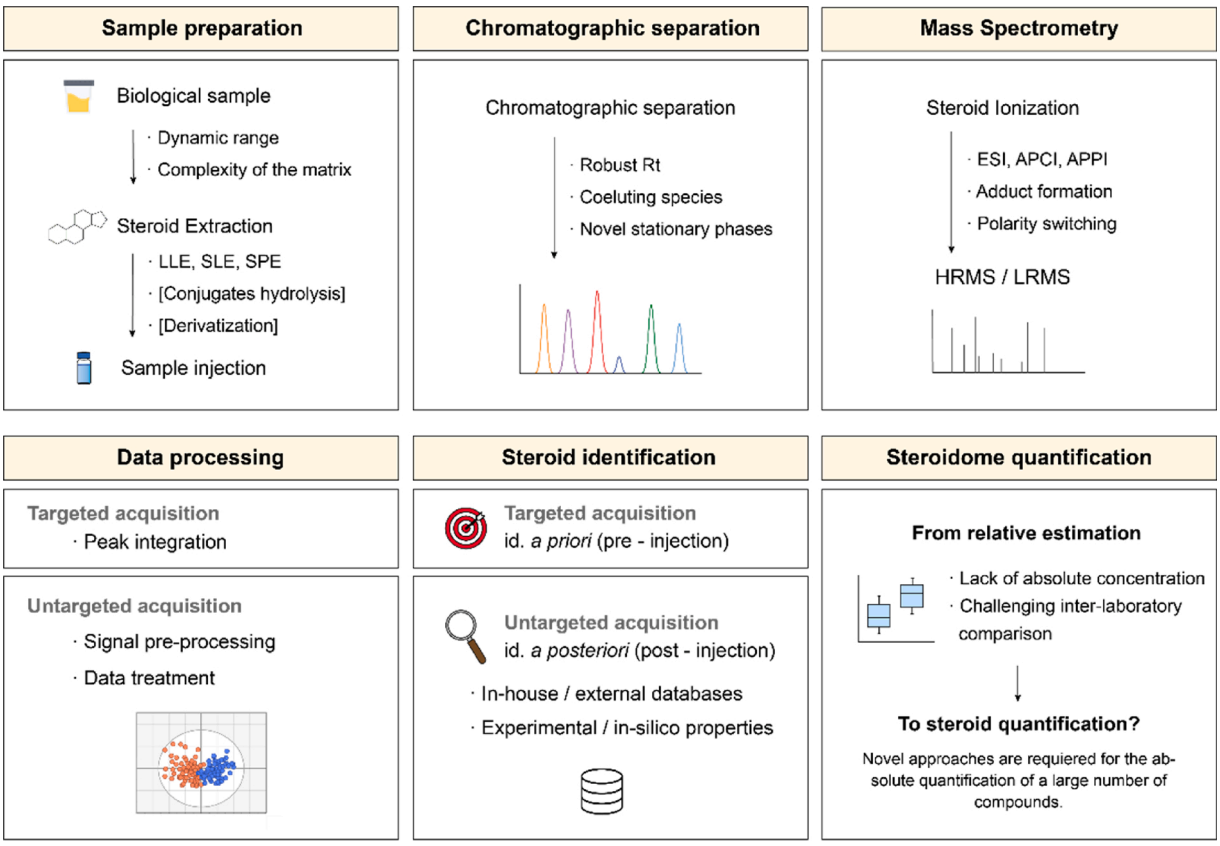

Fig. 5. Schematic representation of the steps for steroidome analysis (sample preparation, chromatographic separation, mass spectrometry, data processing, steroid identification and steroid quantification).

extensive signal pre-processing and peak annotation steps before results can be interpreted in a biological context [143].

The annotation of steroids in biological samples using untargeted acquisition is commonly done either by means of in-house databases that are built with experimental retention times using commercial or synthetized standards, or by using predicted retention times (such as those obtained with DynaSti, a publicly available retention time prediction tool) [55]. However, one of the current limitations of comprehensive steroidome analysis is the unequivocal compound identification. Indeed, due to the closely related structures of the steroids, it is not uncommon to find many compounds presenting the same masses, fragmentation patterns and retention times and cannot be fully distinguished. Thus, different chromatographic peaks could putatively correspond to the same chemical entity, and there are cases where a peak can correspond to several different steroid molecules. These challenges of steroid identification can be mainly caused by i) a spectral overlap due to incomplete chromatographic separation (i.e., isomers), ii) a poor signal-to-noise ratio for low-intensity peaks (low-abundance steroids), iii) a lack of reference LC–MS spectral data for metabolites (and commercial standards), and/or iv) the presence of spectral artefacts (such as interferences or metabolite by-products) that hamper steroid identification [144].

Finally, a balance must be found between validated methodologies allowing for a low number of compounds to be quantified precisely and accurately, and those established for profiling and generic strategies providing a larger coverage but only relative information of the compound concentrations [145]. Currently, the simultaneous study of a large number of steroids is limited to the comparison of the relative estimation of each compound between samples or experimental groups, and achieving a greater level of quantitative detail would depend on the context-specific information required. In this way, it seems that today, steroidomics is mainly designed for initial exploratory purposes, potentially providing a selected set of steroids that could be further investigated using optimized protocols for quantification [138,145].

4. Challenges and limitations of steroid quantification

Overall, quantification is crucial to determine physiological concentration ranges (or reference clinical intervals) allowing to distinguish normal variations from pathological levels. In this context, it must be kept in mind that steroids concentrations are submitted to strong variability from biological factors such as physical activity, sex, age, stress, or diet [18,146,147]. In particular, certain steroids such as adrenal and estrogenic hormones are deeply affected by circadian and menstrual rhythms, and the latter must be taken into account during sample collection to minimize their contribution as confounding factors [148]. A number of strategies are widely adopted to reduce the impact of biological cycles in the analytical results, such as urine collection over 24 h to neutralize circadian fluctuations [18].

One of the limitations when trying to quantify a high number of steroids is the lack of reference materials (standards and/or isotopically modified standards) available for many compounds (especially for phase II metabolites [18]). Thus, several research efforts have been made to synthesize steroid standards, which are expected to contribute to the feasibility of the comprehensive quantification of the steroidome [149,150]. For measurements performed with absolute quantitative purposes, it is essential to rely on the use of internal standards. If the number of quantified analytes makes it possible, the use of commercially available isotopically labelled standards matching the quantified steroids is the option of choice (normally ^{2}H or ^{13}C isotopically labelled analytes). Choosing ^{2}H or ^{13}C internal standards will be mainly a matter of availability. Since ^{13}C-labelled compounds provide better retention time matching to their unlabelled counterparts than deuterated ones, they are the best choice when equal retention times are required to minimize

matrix effects at the ionization step (i.e. ion suppression or enhancement).

Another key factor that challenges the quantification of steroids is the lack of a clear normalization strategy for some biological matrices such as breast milk, saliva or seminal fluid [21]. The diversity in volume, density or viscosity of the samples challenges the quantitative analyses and, so far, there is no universal strategy allowing to normalize the results. Probabilistic quotient normalization (PQN) works particularly well since it does not depend on an external parameter, but on the intrinsic intensities of the set of measured compounds in each sample [151]. The use of ratios of steroids linked by consecutive enzymatic reactions rather than their individual concentrations circumvents the normalization problem and is extremely useful in clinical diagnosis when enzymatic disfunctions are suspected [97,144].

An additional challenge for comprehensive quantification relies on the recovery extraction of the whole steroidome of a sample and simultaneous steroid measurement in the instrument. Currently, a comprehensive steroid detection on MS instruments with a single sample preparation strategy and a single chromatographic methodology remains unfeasible. Complementary extraction procedures, chromatographic strategies and mass spectrometric approaches are and will certainly be needed for achieving steroidome quantification.

The absence of analyte-free biological matrices hampers the quantification of endogenous compounds. This issue is commonly addressed by using other calibration strategies (such as standard addition or background subtraction and surrogate analyte/matrix, among others [152,153]). For steroids quantification, different research groups are developing original strategies aimed at enabling the high-throughput analysis of a large number of compounds without increasing the overall analytical effort. As an alternative to the use of analyte-free matrices, some quantification approaches rely on isotopically labelled compounds surrogate analytes to build the calibration function [156]. New calibration approaches are evaluated to reduce the analysis time while maintaining precise and accurate quantification [154,155]. These solutions could be limited for steroid quantification due to the wide dynamic range of the compounds present in the biological matrix. Further studies on this aspect will facilitate a faster screening of steroid alterations in biological matrices, providing a more comprehensive quantification of the steroidome.

5. Conclusions

During recent decades, advances in methodology and technology have contributed to extending the quantification of a single steroid to that of a large panel of compounds. In many examples, the potential of simultaneously covering a large panel of steroids has demonstrated its utility in contributing to the diagnosis, monitoring and treatment of endocrine pathologies among many other applications. Despite the challenges that present steroid analysis by LC–MS, it remains the technique of choice for the quantitative measurement of either a single steroid or an extensive panel of steroids. The lack of a complete map of the whole steroidome and their endogenous presence in biological samples impacts their quantification. However, many advances in technology and *in silico* strategies would certainly contribute to getting closer to reaching this goal. In this review, we suggest the study of steroidomics as an exploratory approach guiding the later application of a targeted quantification strategy.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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