

Review Article

Estimation of Steroid Hormones in Biological Samples Using Micro Extraction and Advanced Chromatography Techniques

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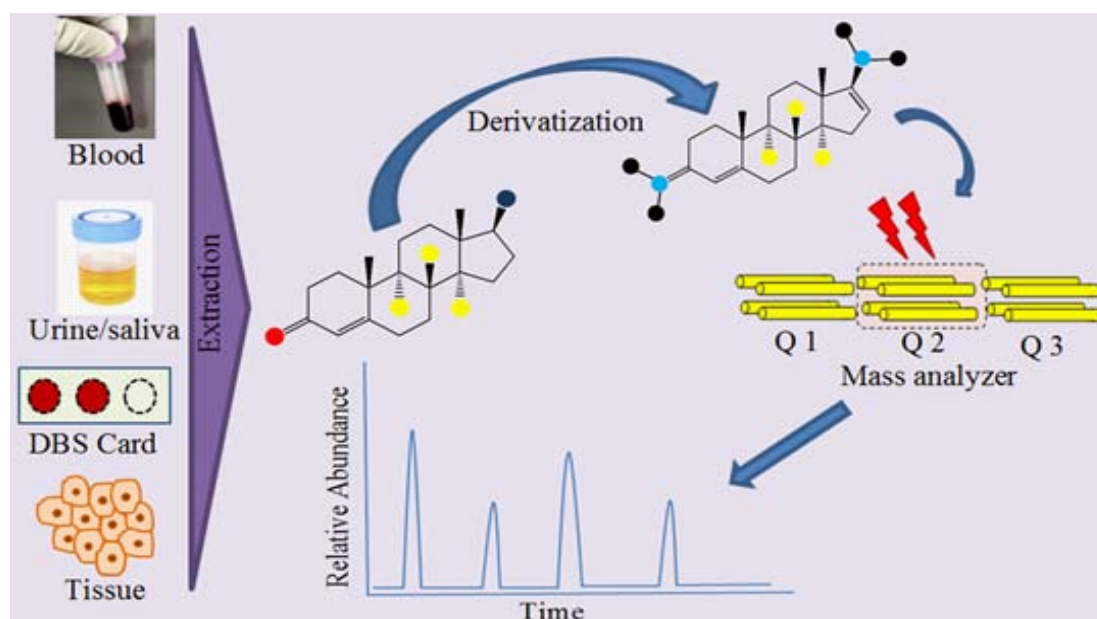
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Abstract

Steroid profiling plays an important role in the clinical setting and also in the diagnosis of various physiological disorders. Steroids in the research laboratory and patient care has been routinely measured by immunoassay. Limitations of immunoassay in quantifying steroids have been well documented and the advent of advanced mass spectrometry is offering a viable alternative to measure multiple steroids in single reaction. Analytes ranging from the steroid and their metabolites that are present in the body carry out various important functions and are relevant to maintaining homeostasis. Blood plasma and urine samples are the clinical material for such analysis, however, due to the difference in polarity of the target steroid, a major challenge exists as to how faithfully analyze different steroids in the given sample type. These steroids are usually present at very low concentrations in the body and are present in several forms leading to increased complexity. Achieving an excellent chromatographic separation for the analysis of steroids requires an effective sample preparation and analysis procedure. Hence various methods are developed to analyze the presence of steroids and to know their exact concentration in the body. This review covers various analytical methodologies such as chromatographic procedures and downstream mass spectrometry for the identification and measurement of the levels of steroid hormones. At the same time, this review also covers the most recent developments that have taken place in the recent past to cover the enhanced understanding of steroid hormone analysis.

Keywords: Steroid Hormone; Gas Chromatography-Mass Spectrometry; Liquid Chromatography-Mass Spectrometry; Sample extraction procedure



Graphical Abstract: Schematic representation of steroid analysis using mass spectroscopy.

Abbreviations

BSTFA, **N**, **O**-Bis(Trimethylsilyl)Trifluoroacetamide; **DBS**: Dried Blood Spot; **DHEA**: Dehydroepiandrosterone; **DHT**: Dihydrotestosterone; **GC-MS/MS**: Gas Chromatography-Mass Spectrometry; **LOD**: Limit of Detection; **LOQ**: Limit of Quantification; **LC-MS/MS**: Liquid Chromatography-Mass Spectrometry; **LLE**: Liquid-Liquid Extraction; **MALDI**: Matrix-Assisted Laser Desorption Ionization; **MSTFA**: **N**-Methyl-**N**-(trimethylsilyl)trifluoroacetamide; **17-OHP**: 17 α -Hydroxyprogesterone; **SPE**: Solid Phase Extraction; **UPLC**: Ultra Performance Liquid Chromatography; **UPLC (QTOF) MS**: Ultra Performance Liquid Chromatography Quadrupole Time-of-Flight (QTOF) Mass Spectrometers; **UHPSFC-MS/MS**: Ultra-High Performance Supercritical Fluid Chromatography-Tandem Mass Spectrometry

Introduction

Steroid hormones play a significant role in regulating different body functions, including salt and water balance, immune response, ability to endure illness and injury, and development of secondary sexual characteristics [1-3]. Steroid hormones are a biologically active group of molecules derived from cholesterol [4]. Any dysregulation in the biosynthesis, metabolism and excretion of steroids could lead to an endocrine disorder. Endocrine disease which is associated with the alteration of steroids can be diagnosed by the quantification of steroids and the ratio analysis of their specific pathway products.

The hyphenated chromatography technique, coupled with mass spectrometry, is gradually becoming a significant approach for the quantification and identification of steroid hormones in biological samples [5,6]. Fast analysis, improved selectivity, and the need for only a small sample volume make this type of technique superior in comparison to traditional and routinely used immunoassay methods [7]. Immunoassay methods are selective and helpful in the detection of a single steroid hormone. However, the robustness of the data, accuracy, and reproducibility of the immunoassay process is not high due to a lot of matrix interference and batch-to-batch variation in the antibodies [8,9].

Liquid Chromatography-Mass Spectrometry (LC-MS/MS) and Gas Chromatography-Mass Spectrometry (GC-MS/MS) are advanced chromatography approaches in the field of steroid hormone analysis [10]. Liquid chromatography-mass spectrometry is an excellent tool in comparison to GC-MS/MS in respect of the determination of individual steroids, but in the case of metabolite identification, GC-MS/MS shows a better response [11,12]. A lot of research studies have been undertaken on the identification and quantification of steroids by using the above chromatography techniques, but only few of those studies are robust and reliable [10,13]. The additional recent analytical approach that has become more prevalent in the analysis of steroids over the past few years is Ultra-High Performance Supercritical Fluid Chromatography-Tandem Mass Spectrometry (UHPSFC-MS/MS) and Matrix-Assisted Laser Desorption Ionization (MALDI-MS). These chromatography instruments are designed to resolve numerous challenging retention/separation problems.

Biological samples are complex and are not directly compatible with mass spectrometry. Therefore, a fast, selective, effective, and robust analytical sample extraction procedure is always important

before the chromatographic separation and analysis of biological samples [14]. Generally, sample extraction is a time-consuming procedure and needs to be highly accurate. Sample extraction has conventionally been executed using Liquid-Liquid Extraction (LLE) [15,16], Solid-Phase Extraction (SPE) [17,18], and Protein Precipitation (PP) [9]. The manual operations associated with these techniques are labor-intensive and time-consuming. Modern trends in sample extraction procedures include Supported Liquid Extraction (SLE), Dried Blood Spot (DBS) [20,21], and Dispersive Liquid-Liquid microextraction (DLLME).

The measurement of steroid hormones is generally performed for public health assessment, patient care, diagnosis and clinical research. All steroid hormones are derivatives of cholesterol which is generally a waxy substance present in blood and animal tissue (**Figure 1**). The transportation of steroid hormones in bloodstream takes place with carrier globulin proteins in a bound form. Abnormalities in the production and metabolism of steroid hormones could potentially lead to various endocrine disorders. For the diagnosis, and treatment of endocrine-related diseases, the absolute quantification of steroid hormones and their metabolites is necessary. In this review, contemporary development in conventional and modern approaches for sample extraction for the analysis of steroids using chromatographic separation and mass spectrometry analysis have been drawn and reviewed. Attention has also been placed on different modern mass spectrometry instruments for the quantification of steroids in different biological samples.

Advanced Sample Preparation Techniques

Sample preparation is a crucial step for the proper extraction of analytes (**Table 1**). To quantify steroid hormones by using a mass spectrometry-based technique, a simple, effective, and robust sample preparation step is necessary to reduce the high matrix effect for better chromatographic separation [22,23]. The extraction techniques vary in terms of sample preparation and also have varying degrees of selectivity, time consumption, and convenience, all of which have a direct effect on the outcome of the final analysis.

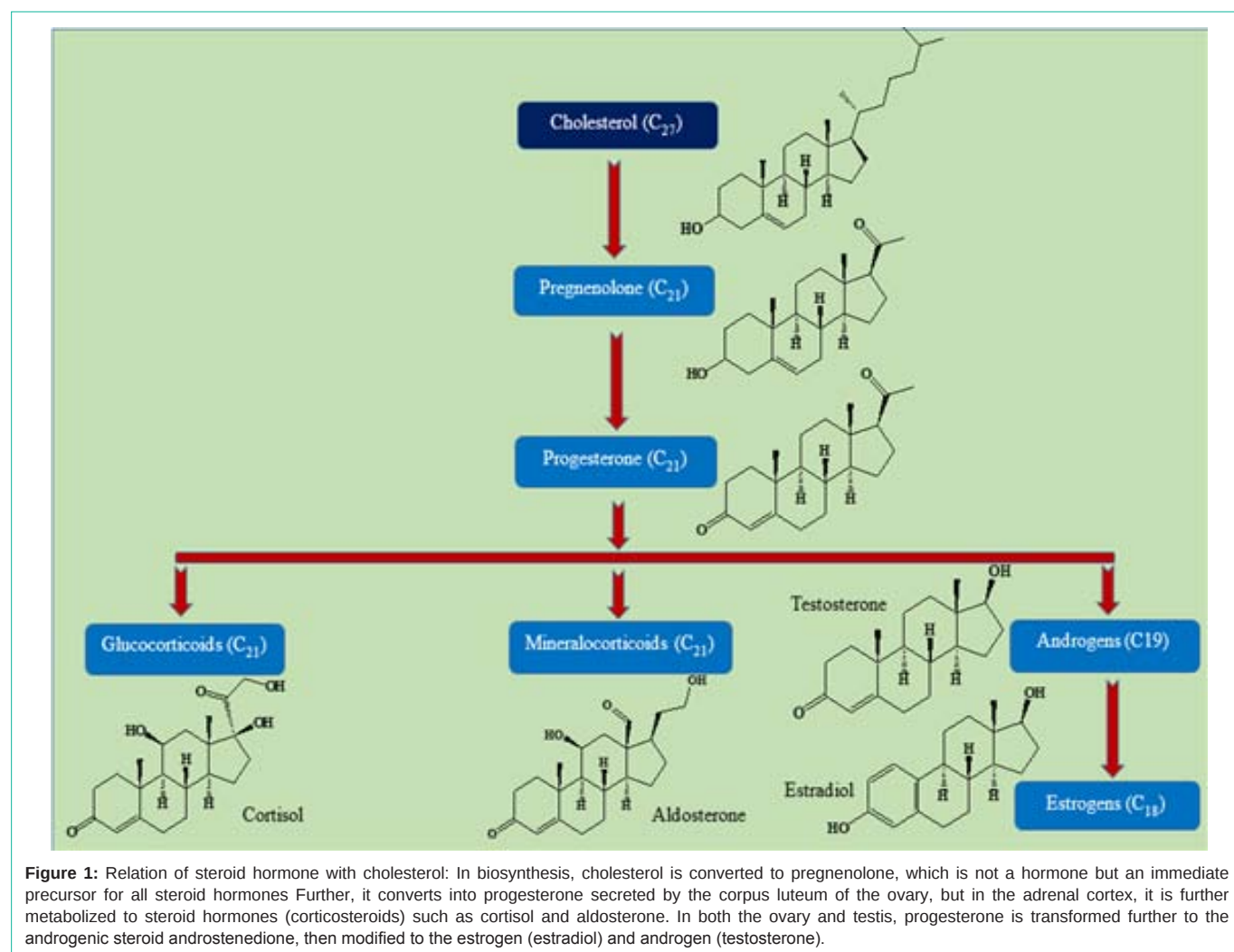
Solid-phase extraction (SPE)

Solid-phase extraction is a highly used sample preparation and pre-treatment approach, especially for trace analytes. In comparison to other extraction procedures, it has a high recovery and robustness to achieve an effective preconcentration and consumes less solvent [24,25]. The selection of solvent and sorbent is a crucial factor in the SPE process. The range of sorbents for SPE varies from graphitized carbon to chemically bonded C8, and C18 silica, and different kinds of polymeric material among others [26,27]. Although SPE has many advantages, it also has some demerits such as its high cost and batch-to-batch variation that affects reproducibility [28-30].

Quantification of steroids in urine samples by using solid-phase extraction on Oasis HLB sorbent and analysis was performed by UPLC-QTOF-MS. The developed method showed good linearity and recovery values, and the Limit of Quantification (LOQ) was in the range of 2 to 500 ng/ml [31]. Another study on the determination of steroid hormone in pediatric blood samples using LC-MS/MS found that the LOD of the developed method was 0.1 nmol/L. The gonadal steroids were extracted by using SPE Oasis MAX elution plates. Before

Table 1: Advantages and disadvantages of the extraction technique.

S.No	Extraction technique	Advantages	Disadvantages
1	Solid-phase extraction	Easy automation, high recovery, low reagent consumption	Low reproducibility, costly, time-consuming, and many procedure steps
2	Liquid-liquid extraction	Easy operation, low extraction cost, simple instrumentation	High solvent consumption, laborious, time taking
3	Protein precipitation	Low solvent consumption, one-step reaction, high reproducibility	Removal of protein is not possible, the high matrix effect
4	Dried Blood Spot	Easier to transport, less blood needed, cost-effective	Storage problem, the possibility of contamination
5	Supported liquid extraction	Faster in comparison to LLE, effective for biological samples	Costly, time-consuming
6	Dispersive liquid-liquid microextraction	Low solvent requirement, fast analysis,	long extraction time, limited solvent choice



the extraction procedure SPE plates were conditioned with 200 µl of methanol after that acidified samples were loaded. Targeted analytes were eluted using 60 µl isopropanol, followed by 100 µl high-purity grade water [32]. For the analysis of estrone and its metabolites, a rapid and effective method was proposed, that utilized the LC-MS/MS technique, and the developed method showed good accuracy between 88% and 108% [33].

The use of tandem mass spectrometry and hydrophilic interaction liquid chromatography separation in combination with SPE extraction techniques also helped in analyzing the seven conjugated

estrogens in urine. This approach produced a linear dynamic range of three orders of magnitude, 92-109 % recovery, 84-109 % intraday accuracy, 1-14 % intraday precision, 80-111 % inter day accuracy, and 1-22 % inter day precision [34].

Liquid-Liquid Extraction (LLE)

Liquid-liquid extraction is based on the separation of targeted analytes from one phase of solvent to another phase of another solvent based on polarity [35,36]. This technique can be used to extract nonpolar steroid hormones from serum or plasma into an organic solvent. Generally, one solvent is an aqueous medium and

the other solvent or extraction solvent is non-polar resulting in phase separation [37,38]. In some LLE extraction protocols, sodium chloride is also mixed in the aqueous phase to aid the allocation of analytes into the organic phase, the procedure known as “salting out”. Extraction solvents having lower density in comparison to the aqueous medium may be separated by using a handheld pipette.

Liquid-liquid extraction is widely used for the quantification of steroids in different matrices. A study that was performed to determine the level of aldosterone in serum using methyl tertbutyl as an extraction solvent followed by LC-MS/MS system reported that the LOD and LOQ of the developed method were 22 pmol/L and 50 pmol/L, respectively [39]. Another study used the combination of hexane and ethyl acetate at a ratio of (3:2) for the quantification of T, DHT, E1, and E2 in serum samples using LC-MS/MS in the APPI mode [40]. In the LLE procedure pH play an important role, the use of an acidic or basic pH is vastly suggested so that contaminations such as phospholipids and cholesterol esters are removed. The measurement of T and DHT in serum samples using LLE as an extraction procedure required the adjustment of pH of the organic phase by adding 350 μ L of 0.1 mol/L sodium hydroxide followed by LC-MS/MS in the ESI mode. The developed method showed good recovery for T and DHT of 100 to 113% and 98 to 107%, respectively [41]. The same extraction process was applied for the quantification of T in serum samples. The proposed method was found to provide high accuracy and excellent precision with a lower detection value LOD of 9.71 pmol/L [42].

Protein Precipitation (PP)

Protein precipitation is one of the simplest and most traditional sample preparation techniques for the extraction of analytes from biological material. Due to the effectiveness of the PP technique, it has been used in many recent applications [43]. The quantification of 12 steroid hormones in human serum samples was developed by using the PP method. The LOQ of the developed method was in the range of 0.005 ng/ml for estradiol to 1 ng/ml for cortisol. The average recovery of the developed method was in the range of 86.4 to 115.0% [44]. To determine 14 carbonyl-steroid hormones in human serum samples after protein precipitation, 2-hydrazinopyridine was used as a derivatization reagent. The correlation coefficient (R^2) of the developed method was in the range of 0.9885 to 0.9998, and LOD was in the range of 0.07 to 65.25 ng/ml [45]. Doping analysis has been recognized as a challenge for testing laboratories. The present study focused on the analysis of steroids for doping control by using PP and the LC-MS/MS instrument. The method was validated by using 50 μ L of human plasma and was found to show a functional recovery of between 80 to 112%, and at LOD in the range of 0.1 to 10 ng/ml [46]. Laura J. Owen et al. also developed a method by using the same extraction technique for the analysis of serum cortisol with LC-MS/MS, in which only 20 μ L of serum sample was used. The result showed a good R^2 value (0.999), and the LOQ of the developed method was 12.5 nmol/L [47]. A method was proposed for the quantification of T, A, and DHEA in human serum samples, which also used LC-MS/MS but which employed a combination of two different extraction procedures, LLE and PP, and methanol as an extraction solvent. The result showed that the LOD of the targeted analytes T, A, and DHEA was 0.22, 0.22, and 1.08 nmol/L, respectively [48].

Dried Blood Spot (DBS)

A dried blood spot type of bio sampling in which a blood sample is sucked onto a DBS card or filter paper and dried [49,50]. Dried blood spot sampling is an effective process, especially for screening neonates for metabolic disorders [51,52]. The collection of a blood sample through DBS is normally conducted by pricking the finger, heel, or toe with a lancet. A minimal quantity of typically 20 to 30 μ L of blood is evenly spread across the spot. The sample is collected by punching out the spotted area and performing extraction by using a suitable extraction buffer or solvent (methanol or acetonitrile). After repeating the extraction step twice, the extract is centrifuged, and the supernatant is collected, dried in a concentrator, reconstituted in a suitable solvent, and then analyzed by using a chromatography instrument [53]. Most often, GC-MS/MS, UPLC, or LC-MS/MS are used in the DBS analysis [54-56].

A precise method was developed for the quantification and profiling of steroids in neonates by using an advanced DBS technique followed by LC-MS/MS. The R^2 value of the developed method was greater than 0.99, and the LOQ for all steroids was in the range of 0.9 to 40.0 nmol/L. the developed method was successfully applied to quantify nine steroids in DBS for clinical diagnosis of congenital adrenal hyperplasia [57]. Recently, a study of neonatal screening for congenital adrenal hyperplasia with secondary steroid hormones using LCMS/MS was conducted. The method was developed by using diethyl ether as an extraction solvent combined with the DBS technique [58]. In another DBS-based study, seven steroid hormones were analyzed in the Korean population. The DBS card procedure was used for blood collection, and in the extraction step, 200 μ L of 50% methanol + 50% acetonitrile was used. The proposed method shows good selectivity and sensitivity. The LOQ of all steroids is in the range of 0.5 ng/ml to 1.0 ng/ml [20]. A study was performed for screening of steroids in neonatal using UPLC-MSMS with ESI positive mode. The method was developed for five steroids, which shows recovery >64.1%. LOQ of the developed method was in the range of 2.8nmol/L to 4.3 nmol/L and had a short run time (1.25 min) [59].

Supported Liquid Extraction (SLE)

Supported liquid extraction (SLE) is similar to customary LLE in that it uses the same water-immiscible solvent methods to extract analytes from aqueous solutions, but the aqueous phase is coated onto a high surface area material typically diatomaceous earth within tubes, much like solid-phase extraction. In SLE, instead of shaking the two immiscible phases together as in LLE, the aqueous sample is immobilized on an inert carrier and the organic phase is flushed through the carrier matrix to extract the target analyte [60].

Recently, SLE has been used as an alternative to LLE and SPE for different biological matrices [61]. To date, several research papers have provided evidence of the utility of this method and its effectiveness as an alternative to methods such as SPE, PP, and LLE [62]. SLE clean-up provides an efficient recovery and decreased background noises. An SLE-based method for the analysis of hydrocortisone in mouse serum using LC-MS/MS was devised. The quality control samples' intra and inter-day precision and accuracy at low, medium, and high concentration levels revealed > or =12.9% CV and 3.4-6.2 percent bias for the analyte in mouse serum [63]. Steroid profiling is used in clinical trials, but there is always room for improvement. Methods

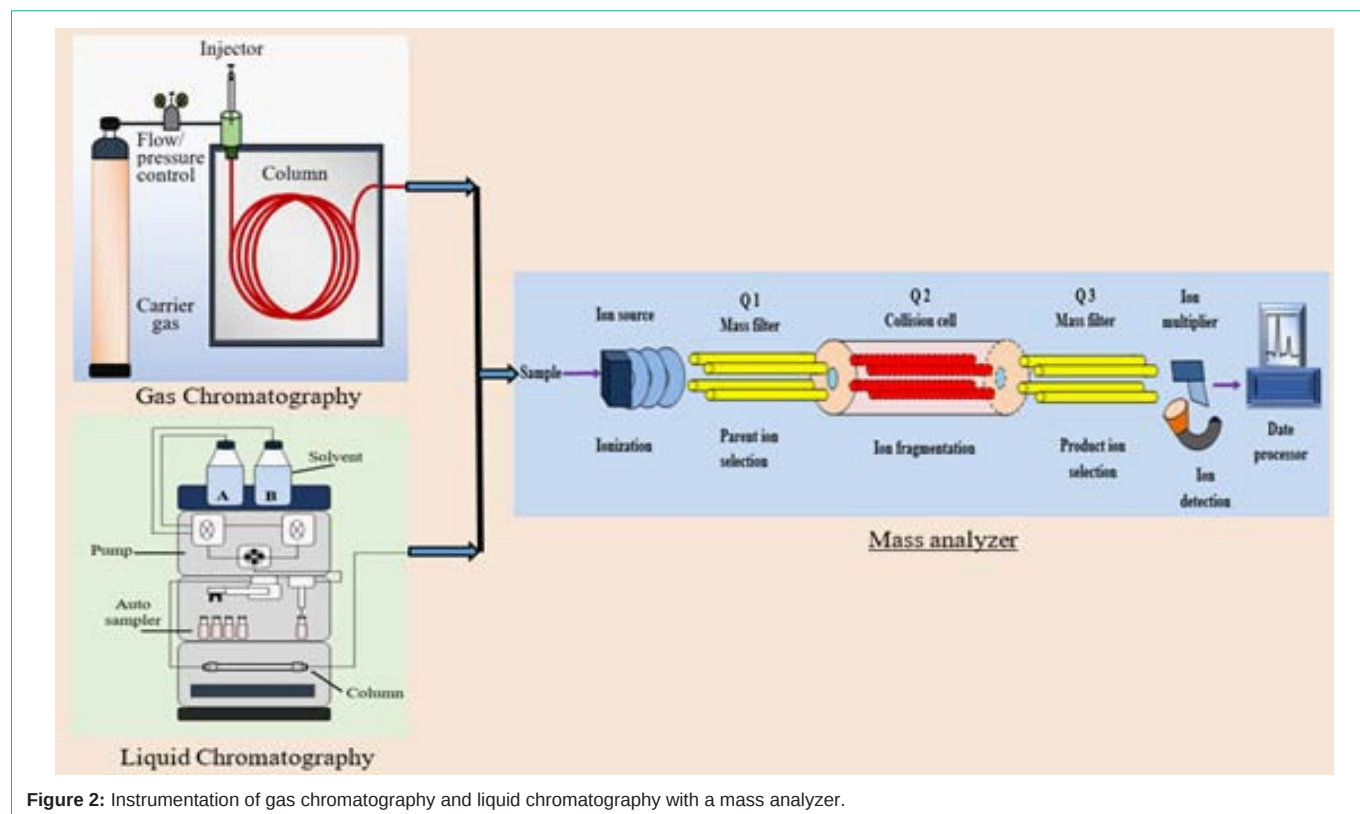


Figure 2: Instrumentation of gas chromatography and liquid chromatography with a mass analyzer.

have been developed that include SLE technology and GCMS/MS that combine Selected Reaction Monitoring (SRM) with selected ion monitoring modes. Here, the LOD of serum steroids ranged from 0.2 to 5 ng/ml and R² was higher than 0.999. Accuracy and accuracy ranged from 1.4 to 10.5% and 82.7 to 115.3%, respectively. The overall recovery of the 30 steroids ranged from 62.1% to 104.3%, while the recovery of the 7 sterols ranged from 44.7% to 75.7% [64].

Dispersive Liquid-Liquid Micro Extraction (DLLME)

Dispersive liquid-liquid micro extraction is a novel sample extraction procedure offering a high enrichment factor for the targeted analyte [65]. In the DLLME extraction procedure, extraction of the targeted analyte takes place in the dispersion of the extracting solvent prepared in an aqueous medium. The whole extraction procedure can be divided into two steps. In the first step, the mixture of extraction and disperser solvent is forcefully injected into the aqueous medium which leads to the formation of a cloudy solution and the second is the removal of the dispersed analyte by centrifugation. The extraction solvent containing analytes is suspended in the centrifuge tube and taken for analysis by using a micro syringe. This extraction process is associated with various advantages like selectivity, simplicity, low cost and high enrichment efficiency. Due to its high efficiency, DLLME is widely used in different environment toxicant analyses like pesticides [66-68], PAHs [69,70], and pharmaceutical products [71,72]. DLLME also deliver an advantage for the analysis of steroids analysis in biological samples. The extraction procedure for the isolation and pre concentration of 17 β -estradiol, in human urine samples has been developed. A mixture of carbon tetrachloride and ethanol (1: 5; v / v) used as an extraction or dispersion solvent was quickly infused into a 2 ml urine sample with 8% NaCl added. The proposed

method provides an efficient method for the analysis of estradiol in human pregnant urine samples [73]. To make this extraction method more effective, DLLME and Hexafluoroisopropanol (HFIP) -alkyl carboxylic acid high-density super molecular solvent for extracting steroid sex hormones in human urine samples using HPLC MS / MS. A study using a combination of SUPRAS was proposed. In this process, octanoic acid was used as the extraction solvent and HFIP was used as the dispersion solvent. The detection limit of the developed method based on the signal-to-noise ratio of 3 was 0.010.10 μ g / L. The recovery rate was 82.7-120.2% [74].

Advanced Chromatography Techniques

Role of mass spectrometry in the measurement of steroids

Mass spectrometry techniques have been utilized extensively in biomolecule identification, steroid analysis, and in chemical and the agricultural industry [75,76]. A predominant technique for steroid analysis based on mass spectrometry is gas or liquid chromatography (GCMS / MS or LCMS / MS) combined with mass spectrometry (**Figure 2**). Mass spectrometry is based on the movement of charged particles in an electric and magnetic field [77]. Only charged particles can be analyzed by using this technique, so the mass-to-charge ratio (m/z) is very important for the effective motion of a particle of interest [78,79]. Additionally UHPSFC-MS/MS and MALDI-MS are also discussed in this review.

Liquid Chromatography-Mass Spectrometry (LC-MS/MS):

In recent years, the importance of LC-MS/MS has increased due to its specificity, high precision, selectivity, and accuracy compared to immunoassays. The main advantage of LC-MS/MS is that it can quantify a range of steroids in a single run. Liquid chromatography-

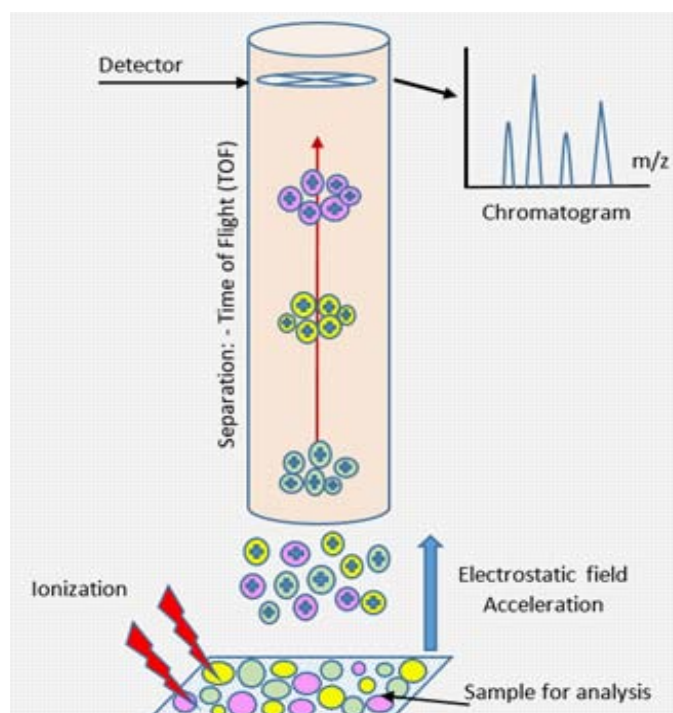


Figure 3: Schematic representation of matrix-assisted laser desorption/ionization (MALDI).

mass spectrometry is a powerful analytical technique that is changing how steroids are analyzed in the clinical laboratory [80].

An overview of steroid analysis using different sample preparation techniques with LC-MS/MS is provided in Table 2. Comprehensive steroid hormone profiling is crucial for monitoring the occurrence and expansion of many related diseases [81]. A new LC-MS/MS method that was combined with an extraction procedure was proposed for the quantification of 17-OHP, AD, and T steroids in plasma. These steroids play an important role in the diagnosis and monitoring of hyperandrogenic disorders. The LOD of the developed method was in the range of 0.03 to 0.06 $\mu\text{g/L}$ [82]. To quantify multiple steroid hormones in a single run, a rapid quantification method was proposed for the quantification of 13 steroids in human plasma by the combination SPE with LC-MS/Q-Trap. The LOD of the developed method was in the range of 0.02 to 9 $\mu\text{g/L}$ [83]. For routine clinical analysis, an advanced method was developed for the analysis of 17-OHP in human serum samples [13]. The quantification of pseudo-endogenous steroids in sports is very important. LC-MS/MS presents an extensive variety of evaluations in human serum, plasma, endometrium, and endometriotic tissue [84,85]. Similarly, a highly sensitive, effective, and easy quantification method was reported for the analysis of estrogen and progestogens in human serum samples. The developed method was found to be helpful for the analysis of both synthetic and endogenous sex-steroid hormones [86]. A comparative study was performed for postmenopausal women to examine the effectiveness of the analysis of estrogens in serum and plasma in a female breast cancer patient by using LC-MS/MS [87]. Postmenopausal women with elevated plasma or serum estrogens are considered to be at increased risk of breast and endometrial cancer, therefore an ultrasensitive quantification method exist using LCMS/

MS [88,89].

Liquid chromatography-mass spectrometry techniques provide an effective quantification method for the analysis of salivary steroids also. Recently a multiplexed analysis method for the analysis of steroid hormones in saliva was developed by combining the LLE and LC-MS/MS techniques. In this method, tannic acid was introduced as a new PP reagent for LC-MS/MS analysis in saliva samples [90]. Another method reported the use of an LC-MS/MS method for the quantification of salivary testosterone in adult males and females. The developed method was successfully applied for the analysis of 104 male and 91 female samples [91]. An alternative utilization of the LC-MS/MS technique using SPE as the extraction method was proposed for the diagnosis of late-onset hypogonadism which is a male-specific disorder caused by the age-related decline of testosterone [92].

Gas Chromatography-Mass Spectrometry (GC-MS/MS):

Gas chromatography-mass spectrometry is widely used in the measurement of many steroid hormones across a wide concentration range and has provided a great opportunity to improve the standards of steroid analysis (Table 2).

GCMS/MS uses a targeted approach to analyze multiple steroids in a sample. Methods have been proposed to quantify nine major steroid hormones in plasma and serum, including T, E1, P, etc. The accuracy of the developed method was 50-112% [93]. For the analysis of steroid esters in human plasma, a highly sensitive method was developed using GC-MS/MS instrument. The method was optimized for the analysis of testosterone and nandrolone and showed a LOD value of 10 pg/ml. The developed method was validated with a range of concentrations from 100 to 2000 pg/ml [94]. The precision and accuracy of the analysis of different sex steroids in breast cancer

Table 2: Overview of analyzing multiple steroid hormones in biological samples using mass spectrometry technique.

Analyte	LOQ	Sample and volume	Sample preparation technique used	Analytical instrument	Column	Mode of ionization	references
T, DHT, E2, E1	10 pg/ml, 50 pg/ml, 2.5 pg/ml, 1.5 pg/ml	Serum 200 µl	LLE	LC-MS/MS	(Supelcosil LC-8-DB, 7.5 cm× 3 mm, 3 µm)	APPI	[40]
15 adrenal steroids	0.015 ng/mL to 20 ng/mL	Serum 250 µl	PP and SPE	LC-MS/MS	BEH C18 column	ESI	[113]
9 hydroxy-androgens	1.0 pg to 2.5 pg	Serum 200 µl	LLE	LC-MS/MS	Kinetex C18 (Phenomenex, Torrance, CA, USA) 100 mm x 2.1 mm, 2.6 µm, 100 Å column with a C18 guard column (2.1 mm internal diameter)	ESI	[114]
8 cortisol	0.9-40.0 nmol/L	Blood	DBS with LLE	LC-MS/MS	-	ESI	[57]
12 steroid hormone	0.005 ng/ml to 1 ng/ml	Serum 100 µl	LLE and PP	LC-MS/MS	Kinetex® 2.6 µm PFP 100 Å column (100 × 3 mm)	ESI	[44]
E1,E2,E3,T,AD,DHEA, P	0.005 ng/ml for E1, E2, and, E3 0.01 ng/ml for T, P, and AD 0.25 ng/ml for DHEA	Serum 100 µl	LLE	LC-MS/MS	ACQUITY UPLC BEH Shield RP18 column (1.7 m, 2.1 × 50 mm)	ESI	[81]
14 carbonyl-steroid hormones	0.07 to 65.26 ng/ml	Serum 100 µl	PP	LC-MS/MS	ACQUITY UPLC HSS T3 (100 mm × 2.1 mm, 1.7 µm)	ESI	[45]
A, T, DHEA	0.22, 0.22, and 1.08 nmol/L	Serum 100 µl	LLE and PP	LC-MS/MS	Phenomenex C18 column (100mm × 2.1 mm, Luna, 5 µm)	APCI	[48]
A, T, and DHT	0.1 nmol/L	Plasma 100 µl	SPE	LC-MS/MS	Waters Acquity UPLC BEH C18 analytical column of 100 2.1 mm and 1.7 µm	ESI	[32]
Androgens and prostaglandins	0.01 to 2 ng/ml	Human urine 100 µl	SPE and LLE	LC-MS/MS	ACQUITY UPLC BEH C18 (2.1 x 100 mm x 1.7 mm)	ESI	[115]
Steroids including doping agents	0.1 to 10 ng/ml	Human plasma 50 µl	PP	LC-MS/MS	Hypersil gold C18 analytical column (2.1 mm x 50 mm, 1.7 mm)	ESI	[46]
T and 5 alpha-DHT	0.2-40 ng/ml and 0.01-2ng/ml	Serum 300 µl	LLE and SPE	UPLC-MS/MS	Acquity BEH C18	ESI	[116]
Cortisol	12.5 nmol/L	Serum 20 µl	PP	LC-MS/MS	Phenomenex 30 x 2.1 mm C8 Kinetex analytical column	ESI	[47]
F, AD, 17 OHP	10 ug/L	Blood spot	DBS and LLE	LC-MS/MS	Waters C18; (50 mm x 2.1 mm)	ESI	[58]
Seven steroids	0.5 ng/ml to 1.0 ng/ml	Blood spot	DBS And LLE	LC-MS/MS	Kinetex 2.6 µm XB-C18 (2.1 mm×50 mm)	ESI	[20]
17-OHP, AD, T	0.03-0.06 ug/L	Plasma	Online SPE	LC-SMS/MS	Chromolith RP 18	APCI	[82]
11 steroids	-	Plasma 150 µl	LLE	LC-MS/MS	C18, bipheny	ESI	[117]
9 steroid	0.10 to 2.00 ng/ ml	Plasma and serum	SPE	GC-MS/MS	Zebron 5HT I (30 m x 0.25 mm, 0.25 µm)	EI	[93]
15 steroid	0.62 to 2.6 ng/ml	Rat blood	SPE	GC-MS/MS	Varian,VF-5 MS, (30 m x 0.25 mm x 0.25 µm	EI	[118]
T and nandrolone	10pg/ml	Plasma 500 µl	LLE	GC-CI-MS/MS	Agilent HP5MS	CI	[94]
15 estrogens, 6 androgens and 2 progestins	0.180 to 1.25pg	Breast tissue	-	GC-MS/MS	MXT-1 (30 m x 0.25 mm x 0.25 µm)	EI	[95]
22 steroids	0.1 to 20 ng/ml	Urine 1ml	SPE	GC-MS/MS	HP-Ultra1, (16 m x 0.2 mm x 0.11 µm)	EI	[97]
15 steroids	0.05 to 2 ng/ml	Urine	LLE	GC-MS/MS	TR-50MS (15 m, x 0.25 mm, 0.25 µm)	CIP	[98]

Estrogens their metabolites and T	0.25 pg and 2.5 pg	Urine 5ml	SPE	ID/GC-MS/MS	Optima-1-MS, (25 m x 0.2 mm x 0.10 μ m)	EI	[99]
17-BE, 2-MEOE,	18.4 pg mL ⁻¹ and 5.5 pg mL ⁻¹	Plasma 0.5 ml	SPE	GC-MS/MS	ATTM-5MS (30 m x 0.25 mm x 0.25 μ m)	ESI	[119]
40 steroids	$\leq$ 50 nM	Urine 1.5 ml	SPE	GCxGC-TOF MS	RXI-1MS (15 m x 0.25 mm x 0.25 μ m)	EI	[120]
13 steroids	0.025 to 2.50 ng/ml	Plasma 90 μ l	Online –SPE	UHPLC-MS/MS	ProntoSIL 120 C18H (10 x 0.5 mm, 5 μ m)	ESI	[121]
Aldosterone	1 pg	Serum 200 μ l	SPE	LC-MS/MS	Cadenza CD-C18 (150 mm x 3 mm, 3 μ m)	ESI	[122]
E1 and E2	0.5 pg/ml	Serum 0.25 to 1ml	SPE	LC-MS/MS	Cadenza CD-C18 (50 mm x 2 mm, 3 μ m)	ESI	[123]
E1, E2, E3, T, AD, DHEA, and P	0.005 ng/ml to 0.25 ng/ml	Serum 100 μ l	LLE	LC-MS/MS	ACQUITY UPLC BEH Shield RP18 column (1.7 μ m, 2.1 x 50 mm)	ESI	[124]
E1 and E2	1.3 ng/L and 1.2 ng/L	Serum 300 μ l	PP	LC-MS/MS	Monolithic C18 (3x100 mm)	ESI	[125]
37 Steroids	0.2 ng to 5 ng/ml	Serum 100 μ l	SLE	GCMS/MS	MTX-1 (30 m x 0.25 mm x 0.25 μ m)	ESI	[75]
Aldosterone	40 pmol/L	Plasma 400 μ l	SLE	LCMS/MS	Phenomenex Luna C18 (50 mm x 2.1 μ m, 5 μ m)	ESI	[73]
glucuronide and sulfate steroids	0.1 ng/ml to 0.5 ng/ml	urine 2 ml	SPE	UHPSFC- MS/MS	Acquity UPC2 BEH (3.0x100mm, 1.7 μ m)	ESI	[126]
Cortisol, testosterone, and progesterone	1.0 nmol/L to 25 nmol/L	Saliva 300 μ l	LLE	LC-MS/MS	Phenomenex (25 x 4.6 mm) C18 column	ESI	[90]
Testosterone	5 pmol/L	Saliva 200 μ l	LLE	LC-MS/MS	Waters C8 Kinetex 3.0 x 100 mm x 2.6 μ m	ESI	[91]
Progesterone, estrone, 17 β -estradiol, estriol, 17 α -ethinylestradiol	0.01-0.10 μ g/L	Urine 900 μ l	DLLME	HPLC-MS/MS	Sepax BR-C18 column (150 mm x 2.1 mm, 3 μ m)	ESI	[74]

tissue samples using a GC-MS/MS instrument reported a range of 4.2 to 26.8% with an acceptable recovery of 90.8 to 116.4% [95]. Gas chromatography-mass spectrometry is the preferred analytical technique for the quantification of endogenous steroids in athletics. The glucuronide or sulfated conjugated steroid hormones first undergo deconjugation and then derivatization to make them suitable for GC-MS/MS analysis [96].

Urinary steroid hormone profiling is a primary and long-established analytical technique that provides a vast range of information related to a disorder of human steroid biosynthesis and catabolism. The extraction was done by using a C18 SPE cartridge with 4 ml of methanol and 4 ml of water as an extraction solvent. The LOQ value was in the range of 0.1 to 20 ng/ml and the accuracy varied between 80 to 120% [97]. The same study was also performed for the quantification of 15 steroids in urine samples by using a capillary photoionization ion source equipped with GC-MS/MS. The relative standard deviation value was between 5 to 18%, while the LOD and LOQ ranged from 2 to 100 pg/ml and 0.05 to 2 ng/ml, respectively [98]. An advanced GC-MS/MS protocol (benchtop dilution/benchtop gas chromatography) showed good chromatography linearity and reproducibility with R² value between 0.999 to 1.000 % [99].

A lot of research articles have demonstrated the efficacy of derivatization in the analysis of steroid hormones. A comparative

study investigated the efficiency of several derivatization reagents e.g. BSTFA with TMCS, MSTFA, and BSA for steroid hormone analysis using GC-MS/MS. The results showed that MSTFA performed the best in comparison to others in metabolite analysis. A study performed by the same research group targeted the analysis of 12 steroids by separately using BSTFA+1% TMCS, MSTFA, and BSA as derivatization reagents, from which it was concluded that MSTFA was again more efficient in comparison to the other reagents [100]. In contrast, the analysis of E1 and EE2 by GC-MS/MS using MSTFA and BSTFA as a derivatization reagent showed that BSTFA has better performed [101]. On other hand, a rapid microwave-accelerated derivatization method was proposed for the simultaneous analysis of five natural and synthetic estrogenic steroids by using GC-MS/MS with BSTFA+TMCS in pyridine solution as the derivatization reagent. The developed method showed good recovery and reproducibility. The LOD of the developed method was 0.02 to 0.1 ng/L [102].

Ultra-High Performance Supercritical Fluid Chromatography-Tandem Mass Spectrometry (UHPSFC-MS/MS): Ultra-high performance supercritical fluid chromatography-tandem mass spectrometry has the combined resolution power of GC and the high efficiency of UHPLC. Supercritical CO₂ which has high solubility, effective dissolving capacity, and liquid-like density is used as a primary mobile phase. Generally, a UHPSFC system uses liquid organic modifiers of different polarities. When combined with

supercritical CO₂ and a high-range stationary phase it can provide effective and robust data [103].

A comparative study was undertaken to assess the performance of LC-MS/MS and UHPSFC-MS/MS in screening different molecules [104,105]. It was reported that this technique is highly effective for the analysis of clinically relevant steroids [103]. SFC-MS/MS technique is suitable for steroid analysis that covers different structural classes of endogenous steroids. Systematic investigations revealed that ESI is superior to other ionization processes [106]. A comparative study was also performed to examine the analysis capability of GC-MS/MS and UHPSFC-MS/MS in respect of endogenous steroid profiling to analyze glucuronide and sulfate steroids in urine samples and the former showed better sensitivity [107].

Matrix-Assisted Laser Desorption Ionization (MALDI-TOF)

Matrix-assisted laser desorption ionization is a highly sensitive and effective soft ionization technique that utilizes a laser and laser energy adsorbing solid matrix to ionize analytes with the least fragmentation. Although MALDI normally produces significantly fewer multi-charged ions, it is similar to Electrospray Ionization (ESI) in that both procedures are relatively gentle (low fragmentation) ways of getting ions of big molecules in the gas phase (**Figure 3**). The MALDI technique has been used in several studies to analyze steroid hormones. A method proposed for the quantitative analysis of endogenous estrone in human breast cancer cells. The method developed showed good linearity ($R^2 > 0.99$) with a LOQ of 11 f mol. Based on the results, it was successfully applied to monitor changes in estrone levels in MCF7 cells after treatment with an aromatase inhibitor [108]. To analyze the low concentrations of steroids in plasma samples, hydroxylamine was used as the derivatization agent. The resulting LOD ranged from 0.019 to 0.031 nM, the recovery varied from 86 to 108%, and the coefficient of variation (CV %) ranged from 4.59 to 11.90% [109]. In a different clinical context, the MALDI technique is a convenient way to analyze the free form of steroid hormones in saliva samples. In the case of MALDI analysis when traditional organic matrices are used, it is observed that nanomaterials are also used as a matrix to increase the selectivity of the method. Nanomaterials have a high surface area and good adsorption efficiency, furthermore, nanomaterials reduce the interferences and shows the best suitable for small molecules [110].

Challenges in Mass Spectrometry Assays

While mass spectrometry appears to be becoming the gold standard for steroid hormone analysis, there are still some challenges that need to be addressed. The presence of tissue, biological fluids, and their interaction with steroid hormones of interest is a significant consideration when we develop a method for the quantification of a particular steroid. The major use of qualitative analysis is for the detection of synthetic anabolic steroids or their metabolites in the urine of sportspersons [111]. The list of synthetic and prohibited steroids continues to increase and create new challenges in the field of analytical chemistry [112]. Due to the increased specificity and sensitivity of various methods, various new tools are being developed for the analysis of complex spectra that is developed after analysis. The selective algorithm that can help identify new steroid progeny is

crucial for such analysis. The selection of proper spectral analysis in light of high throughput and faster analysis is the key to overcoming the ever-growing complexity of steroid hormone analysis methods and analysis.

Conclusion

Clinical analysis of steroids is essential for the identification of the different hormones that can have adverse effects on human physiology. Several techniques are available, but they are all very costly and time-consuming. Advanced chromatography instruments can produce robust, precise, and high-quality results. In comparison to GC-MS/MS, recent data shows that LC-MS/MS is more effective, faster, and more sensitive for routine laboratory analysis of steroid hormones whereas GC-MS/MS is more effective for steroid metabolite identification and steroid profiling analysis. Taken together, the results suggest that a MALDI-MS-based quantitative approach is effective in quantifying targeted steroid hormones that may reflect the clinical status and pathogenic mechanisms.

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Conflict of interest

The authors declare that they have no conflict of interest.

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