Applications of Mass Spectrometry in Analyses of Steroid Hormones

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Abstract Steroid hormones are endogenous chemicals controlling many endocrinology functions. Mass spectrometry technologies have been applied for analyses of steroid hormones as biomarkers in endocrinology and pathology diagnoses, doping drugs in athletes and racing horses, residuals in food safety concerns, and environmental pollutants in water and sediments. Both liquid chromatography mass spectrometry (LC-MS or LC-MS/MS) and gas chromatography mass spectrometry (GC-MS or GC-MS/MS) are broadly used in research, clinical, pharmaceutical industry, competition sports, food safety, and environmental testing laboratories. Sample preparation techniques, such as deconjugation and extraction, are critical procedures for isolating steroid hormone from sample matrices, including biological fluids, tissues, environmental water and sediments. Chemical derivatization modifies the physicochemical properties of steroid hormone molecules to improve their chromatographic performances and to enhance their sensitivities to mass detection. Chromatographic techniques such as HPLC, UPLC, and GC have direct impact on separation of analytes, MS interface, and analysis throughput. The method sensitivity and specificity of LC-MS and GC-MS depend largely on the analyte status, i.e., easiness of ionization, derivatization, sample matrix, and MS detection mode, e.g., ESI, APCI, APPI, MAILDI, or EI. LC-MS and GC-MS methodologies should be developed and validated following scientific and regulatory guidelines, and the steroid hormones analyses should be standardized.

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1 Introduction

1.1 Steroid Hormones: Chemical Structures and Functions

Steroid hormones are composed of 18–21 carbon atoms, which are bonded together by four fused rings: three cyclohexane rings (designated as rings A, B, and C) and one cyclopentane ring (as ring D), with an exception of estrogens' ring A as a benzene ring. Hydroxyl and/or ketone groups are attached at C_3, C_{11}, C_{17}, C_{20}, or C_{21} positions, and methyl group(s) is attached at C_{18} and/or C_{19} positions of the steroid skeletons, as shown in Fig. 1. These molecules are neutral, unionized, nonvolatile, lipid soluble, and stable during extraction and analysis by mass spectrometry. Originated from cholesterol, the biosynthetic pathways of steroid hormones are well established [1–3]. The major biotransformation reactions for steroid hormones include oxidation, reduction, conjugations with glucuronic acid, sulfate, and glutathione [4, 5]. As most of the biotransformation pathways and metabolites of steroid hormones are clearly defined, structure elucidation or identification is not a major concern for steroid analysis [6]. However, since a large portion of endogenous steroid hormones exists as their glucuronide and sulfate conjugates, especially in urine, deconjugation of the steroid hormones may be needed before LC-MS/MS and GC-MS analysis [7, 8].

According to bonding receptors and biological functions, steroid hormones are classified as progestagens (or progestogens, pregnancy hormones), mineralocorticoids (mineral retention hormones), glucocorticoids (glucose metabolism and inflammation hormones), androgens (male hormones), and estrogens (female hormones). Endogenous steroid hormones are chemical messengers regulating many life functions, such as controlling pregnancy, salt and water balance, metabolism, inflammation, immune functions, and development of sexual characteristics. Many serious health problems, diseases, and clinical syndromes are indicated by steroid hormones deviations from normal levels. Steroid hormone analysis plays important roles in health care, including disease diagnosis, food safety, and environmental protection. For example, endogenous steroid hormones are critical biomarkers for clinical diagnoses in endocrinology, physiology, and pathology; and steroid hormones are also broadly used as medicines for treatment of varieties of diseases, such as inflammation, malfunctions in immune systems, underdevelopment syndromes, and disorders in endocrine systems [2, 9]. On the other hand, steroid hormones are abused by some athletes [10] and racing horse owners [11] to enhance their sport performances. Steroid hormones are also found as hazardous residuals in eatable meats [12] and are monitored as environmental pollutants in water and sediments [13]. Therefore, development and application of specific, sensitive, and robust analytical methods and methodologies for steroid hormone analyses have significant impacts on clinical analyses, disease diagnoses and treatments, antidoping drug screening, food safety examination, and environmental quality monitoring. A large number of articles have been published on analyses of steroid hormones by mass spectrometry

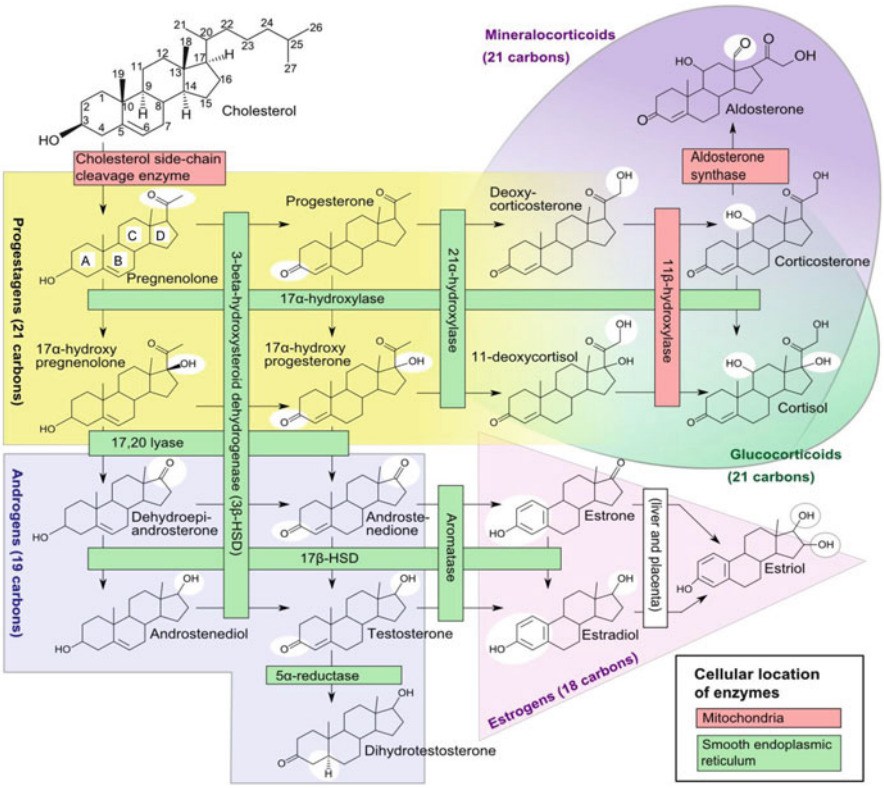

Fig. 1 The major classes of steroid hormones: progestagens, mineralocorticoids, glucocorticoids, androgens, and estrogens. Enzymes, their cellular location, substrates, and products in human steroidogenesis. Boron WF, Boulpaep EL (2003) Medical Physiology: a cellular and molecular approach, p 1300, Elsevier/Saunders (reprinted with permission from Elsevier/Saunders)

and other technologies, and progress and challenges of MS technologies and applications in steroid hormone analyses have been briefly reviewed (see Table 1).

1.2 Challenges to Analytical/Bioanalytical Technologies and Methodologies

Immunoassays (IA) and radioimmunoassays (RIA) are widely accepted and routinely applied for clinical and environmental analyses of steroid hormones, but they have limitations in specificity and accuracy. Therefore, more selective, accurate, and sensitive technologies such as LC-MS/MS and GC-MS/MS methodologies are needed to improve the IA and RIA testing techniques for steroid hormones and

Table 1 Representative reviews on steroids analyses by LC-MS/MS and GC-MS

Steroid analysis	Major topics	Reference
Steroid hormones in clinical chemistry	Role of MS in clinical diagnostic testing and endocrine biomarker analysis; sample preparation, e.g., LLE vs. SPE, and derivatization to enhance sensitivity; LC-MS/MS method validation, specificity, quality management, interpretation of test results for clinical diagnosis; LC-MS/MS analysis of steroid hormones and related physiology/pathology diagnostic significances, including: adrenal steroids, glucocorticoids, androgens and estrogens.	[2]
Steroids hormones in clinical chemistry	Comparison of RIA, GC-MS/MS and LC-MS/MS in steroid analysis; role of LC-MS/MS in metabolomics, e.g., quantitative bioanalysis, identifying and profiling biomarkers; standardization of MS assays.	[14]
Steroids hormones in clinical chemistry	Steroid analysis by LC-MS/MS in pediatrics—challenges in method specificity, sensitivity, and test results interpretation; steroid assays and profiling in plasma, saliva, and urine by LC-MS/MS.	[9]
Steroids hormones in clinical chemistry	Immunoassay vs. GC-MS and LC-MS/MS; derivatization vs. nonderivatization; steroid profiles for newborns, adrenal insufficiency, prostatitis/pelvic pain syndrome, premature adrenarche, sera from smokers, metabolic diseases, diabetes, water contaminant, athletes doping.	[3]
Estrogens in clinical chemistry	RIA, GC-MS/MS, and LC-MS/MS analyses of estrogens in serum and plasma; isotope internal standard; sample derivatization; ionization modes and sensitivities of GC-MS/MS and LC-MS/MS.	[4]
Estrogens in clinical chemistry	Measurement of endogenous estrogens by immunoassays, LC-MS (ESI, APCI, APPI) and GC-MS.	[15]
Endogenous conjugated androgens in clinical chemistry	GC-GS and LC-MS/MS analyses of conjugated androgens—deconjugation, derivatization, and associated issues.	[7]
Steroids hormone residuals in meat safety concerns	GC-MS^n and LC-MS^n analyses of steroids in edible matrices, e.g., meat, liver, kidney, kidney fat, and milk.	[12]
Steroids hormones as environmental pollutants	Analysis of steroids as environmental endocrine disrupting compounds; sample preparation, e.g., LLE vs. SPE; immunoassay vs. GC-MS/MS and LC-MS/MS analyses; sensitivities, e.g., LOD at pg–ng/mL level.	[13]

(continued)

Table 1 (continued)

Steroid analysis	Major topics	Reference
Steroid hormones chemical derivatization, reagent selection and optimization	Derivatization of steroids to enhance LC/MS detection, e.g., $ESI^{+/-}$/MS, $APCI^{+/-}$/MS, and $APPI^{+/-}$/MS.	[16, 17]
Steroid hormones by GC/MS and LC/MS	GC/MS and LC/MS in determination of androgens, corticoids, estrogens, cholesterol and related, bile acids, vitamin D and metabolites, phytosteroids, etc.	[1]
Steroid hormones assay standardization	Immunoassay vs. GC-MS and LC-MS/MS, validation of assay methodologies, establishing standard pools of steroid hormones in women and man, utilizing the pools for cross comparison of various methodologies.	[18]

metabolites [4, 12, 14, 19]. LC-MS/MS and GC-MS methods are increasingly used for analyzing steroid hormones due to their high selectivity and sensitivity. The typical LC-MS/MS methods by direct injection [20, 21] and GC-MS methods [6] have limit of quantitation (LOQ) at ng/mL level, and a number of LC-MS/MS [2, 8, 22] and GC-MS/MS [23] methods are able to achieve LOQ at pg/mL levels when the steroid hormone samples are chemically derivatized before injection. Nevertheless, in many cases, clinical diagnostic tests need to determine steroid hormones at low pg/mL, and even fg/mL levels [4, 15, 18]. In order to improve the MS method sensitivity and sample preparation efficiency, many studies on sample preparation have been carried out, including deconjugation, extraction, and derivatization. Different chromatographic techniques and MS detection modes have also been investigated.

On the other hand, many LC-MS/MS and GC-MS methodologies and their applications to steroid hormone analyses have been developed for the purpose of scientific research or specific studies only, while they might not have been validated according to regulatory guidelines, and their results might not correlate with those from the widely accepted bioanalytical techniques, e.g., IA and RIA. Therefore, standardization of the LC-MS/MS and GC-MS technologies and methodologies, including facilities, instruments, reference standards, procedures, data system, etc., plays a critical role in transferring the LC-MS/MS and GC-MS technologies to daily testing procedures for clinical diagnosis, sports antidoping screening, food safety control, and analysis of environmental contaminations in water and sediments. Only until their specificity, accuracy, precision, calibration mode, sensitivity, and robustness are validated according to regulatory guidelines, and the test results are comparable or consistent with those obtained from the existing techniques such as IA and RIA, the LC-MS/MS and GC-MS/MS techniques may not be accepted as reliable clinical diagnostic methodologies for steroid hormone determination [18].

2 Sample Preparation

The major sample preparation procedures of steroid hormone analyses include extraction, deconjugation, and derivatization, as shown in Fig. 2. A number of examples of steroid hormone sample preparation are summarized in Table 2. Application of stable isotope labeled (e.g., deuterated or ^{13}carbon labeled) steroid hormones as the internal standard or isotope dilution is a standard of practice during quantitative steroid hormone analyses by LC-MS/MS and GC-MS whenever possible. These isotope labeled internal standards are added into the samples before deconjugation or extraction procedures. They undergo the same deconjugation and/or extraction procedures and LC-MS/MS or GC-MS analysis as the steroid samples do. The efficiency of deconjugation and/or extraction, assay accuracy and precision are calibrated and calculated with the isotope internal standards, leading to more accurate, precise, and robust methods and results.

2.1 Deconjugation of Steroid Hormones

A large percentage of steroid hormones exist as glucuronide, sulfate, and glutathione conjugates in body fluids and tissues. These steroid conjugates may be analyzed directly by LC-MS/MS using either electrospray ionization (ESI) [5, 40–43] or atmospheric pressure chemical ionization (APCI) [44] mode. However, in many cases, the presence of glucuronide and sulfate conjugates in samples may reduce MS detection sensitivity and the total amount of a hormone (both unconjugated and conjugated) that can be determined by LC-MS/MS [8] or GC-MS [6] after the

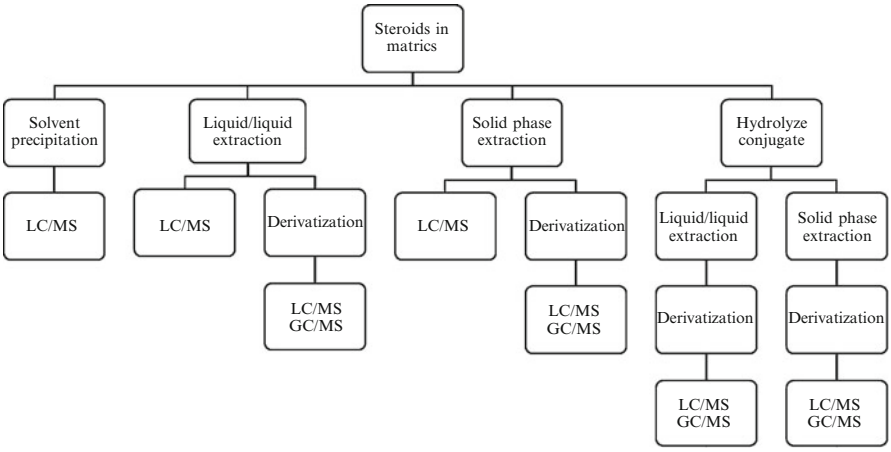

Fig. 2 Steroid hormone sample preparation procedures: extraction, deconjugation, and derivatization

Table 2 Typical steroid hormone sample preparation techniques

Steroids in matrix	Hydrolyzing conjugates	Extraction	Derivatization	Internal standard	MS mode	Reference
Estrogens in serum Steroids in testicular fluid	NA	Solvent precipitation vs. LE LLE	NA	Deuterated isotopes	LC-APPI$^{-/+}$/MS/MS LC-ESI^{+}/MS/MS	[22] [24]
Estrogens in breast tissue, plasma, brain	NA	Solvent precipitation/LLE LLE	Hydroxylamine Dansyl chloride	Deuterated isotopes	LC-TIS^{+}/MS/MS LC-ESI^{+}/MS/MS LC-ESI^{+}/MS/MS	[25] [26] [27]
Steroids in urine Estrogens in urine, serum	β-Glucuronidase/ H_2SO_4 β-Glucuronidase/ sulfatase	LLE LLE	NA Dansyl chloride	Deuterated isotopes	LC-ESI^{+}/MS/MS	[20] [8, 28]
Estrones in cell culture medium Steroids in water	NA	SPE (96-well C_{18}) Magnetic-mediated SPE	NA	Deuterated isotopes NA	LC-APCI$^{+/-}$/MS/MS vs. LC-ESI$^{+/-}$/MS/MS LC-ESI^{-}/MS/MS	[29] [30]
Androgens in prostatic tissue Androgens in plasma Estrogens in sediment and sewage sludge Estrogens in water	NA	SPE (96-well Oasis HLB) Sep-Pak C_{18} Sep-Pak C_{18} Supelco Discovery C_{18}	2-Fluoro-1-methypyridinium *p*-toluenesulfonate (FPMTS) Pentafluoropropionic anhydride BSTFA MSTFA	Deuterated isotopes	LC-ESI^{+}/MS/MS GC-EI^{+}/MS LC-ESI^{+}/MS and GC-EI^{+}/MS GC-EI^{+}/MS	[31] [32] [33] [34]
Estrones in urine	β-Glucuronidase/ sulfatase	SPE (Bobd Elut LRC $C_{18,}$ Extrlut QE)	*p*-Toluenesulfonhydrazide	Deuterated isotopes	LC-ESI^{+}/MS and LC-APCI^{+}/MS, LC-EI^{+}/MS^{n}	[35, 36]
Estrogens in urine	β-Glucuronidase	SPE (Sep-pak C18, Sephadex A25, Oasis HLB)	MSTFA	Deuterated isotopes	GC-EI^{+}/MS	[37, 38]
Steroids in urine	β-Glucuronidase/ arylsulfatase	βCD (Oasis HLB)			GC-EI^{+}/MS	[6, 39]

NA, not applicable

deconjugation and derivatization of the steroid hormones. Thus deconjugation of steroid hormone conjugates become a critical sample preparation procedure in LC-MS/MS and GC-MS analyses.

Both estrogen glucuronide and sulfate conjugates may be deconjugated with hydrochloric acid in methanol [45], but acid solvolysis is not a selective reaction, and the harsh condition may cause side reactions and by-products. An alternative deconjugation procedure applies β-glucuronidase from *Escherichia coli* to hydrolyze steroid glucuronide conjugates first, and then hydrolyzes steroid sulfate conjugates with sulfuric acid [20]. Steroid hormone glucuronide conjugates may be hydrolyzed with β-glucuronidase from different sources, e.g., *Escherichia coli*. [38, 46], limpets [47] or *Helix pomatia* [39]. An attention should be paid to the fact that β-glucuronidase from *Escherichia coli* does not affect the chemical structures of steroid hormones during deconjugation, while β-glucuronidase from *Helix pomatia* can convert 3β-hydroxy-5-ene steroids into 3-oxo-4-ene steroids, and change 3β-hydroxy-5α-reduced and 3β-hydroxy-5β-reduced steroids into 3-oxo-5α-reduced and 3-oxo-5β-reduced steroids, respectively, because *Helix pomatia* also contains other enzymes, including cholesterol oxidase, 3β-hydroxysteroid oxidoreductase/3-oxosteroid-4,5-eneisomerase, and 6-hydroxylase. When glucuronide conjugates of steroid hormones with 3-hydroxy-5-ene structure, e.g., progestagens and androgens, are deconjugated using β-glucuronidase from *Helix pomatia*, the hormone quantitation may not be accurate or representative [48]. However, β-glucuronidase/sulfatase from *Helix pomatia* are commonly used for hydrolyzing both glucuronide and sulfate conjugates simultaneously, especially for estrogens and metabolites, because the phenolic ring A of estrogens is not affected by those enzymes in *Helix pomatia*. The deconjugation of steroid glucuronide and sulfate conjugates is simplified as one step incubation of β-glucuronidase/sulfatase with steroid samples at 37 °C for 20 h or at 55 °C for 3 h [8, 28, 39].

2.2 *Extraction of Steroid Hormones from Biological Matrices, Environmental Water and Sediments*

The techniques used for extracting steroid hormones and metabolites include protein precipitation (PP) with an organic solvent, liquid–liquid extraction (LLE), and solid phase extraction (SPE). As shown in Fig. 2, selection of a sample extraction technique is based on the steroid hormone sample (unconjugated or conjugated), quantity, matrix and the objective of an analytical method or test. For example, if a method or test needs to analyze only the unconjugated steroid hormones in a small volume of serum, e.g., <1 mL, a small volume of acetonitrile can precipitate the proteins and extract steroids from the sample [25]. The procedure is very simple, but the acetonitrile extract contains more nonrelated components than the extract from LLE [22]. When a small volume of biological fluid is extracted by LLE with a solvent, whether the sample undergoes deconjugation and/or derivatization or not, the LLE extract is cleaner than PP extract, but may not be as clean as an extract from

SPE. However, LLE may reduce the sample loss, experiment procedures and errors, and save time in comparison to SPE [8, 25, 28]. In contrast, if a larger amount of sample is available, e.g., 2–10 mL of urine or 1–10 L of environmental water, SPE is a better choice, because it concentrates the sample and minimizes the interferences from other materials, leading to a higher sensitivity and selectivity of the method or test [34, 37, 39]. The sample extraction throughput can be significantly enhanced by using automated 96-well SPE plates [31, 49].

2.3 Derivatization

Chemical derivatization is a standard procedure for GC-MS analysis of steroid hormones, because steroid hormones are not volatile to go through GC column [1, 6]. The major concerns of derivatization reagents for GC-MS analysis of steroid hormones are the completeness of the derivatization reaction and the volatility of hormone derivatives. The typical derivatization reagents for GC-MS samples are silylation reagents, e.g., *N*-methyl-*N*-trifluorotrimethyl acetamide (MSTFA, [37, 39]) and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA, [33]), which react with both alcoholic and phenolic hydroxy groups on steroid hormone molecules.

One of the major advantages of LC-MS/MS over GC-MS or GC-MS/MS is that steroid hormones may be analyzed directly by LC-MS/MS without derivatization procedures, which are time-consuming and tedious [22, 50–53]. However, a number of studies demonstrated that the chemically derivatized steroid hormones were significantly more sensitive to LC-MS/MS detection than the underivatized hormones, because the neutral molecules of estrogens and metabolites might not be effectively ionized under electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) modes [4, 21, 25, 54, 55]. In order to enhance the steroid hormone molecules sensitivity for LC-MS/MS analysis at pg/mL level, chemical derivatization is an effective technique for analysis of steroid hormones and metabolites. A list of derivatization reagents and application examples for steroid hormone analyses by LC-MS/MS and GC-MS are presented in Table 3.

An ideal derivatization reagent is able to react with steroid hormones and metabolites selectively and quantitatively under mild conditions within a short time, and those hormone derivatives are stable and easily ionized during LC-MS/MS analysis. Based on their functional groups, the derivatization reagents used for LC-MS/MS analyses of steroid hormones and metabolites may be classified into seven major classes:

1. Hydrazide, e.g., (carboxymethyl)trimethylammonium chloride hydrazide (Girard T reagent) [4, 56], and *p*-toluenesulfonhydrazide [36]; and hydroxylamine [25]
2. Benzyl bromide, e.g., pentafluorobenzyl bromide [55, 59] and 4-nitrobenzyl bromide [60]
3. Fluorobenzene or fluoropyridine, e.g., 2,4-dinitro-5-fluorobenzene analogues [54] and 2-fluoro-1-methyl-2-pyridinium *p*-toluenesulfonate [61]

Table 3 Typical derivatization reagents and target chemical groups of steroid hormones

Derivatizing reagent	Typical compound and reference	Reaction target	MS mode
Silylation agent	*N*-Methyl-*N*-tryfluorotrimethyl acetamide [6] Pentafluoropropionic anhydride [32]	Both phenolic OH and alcoholic OH	GC-EI$^+$/MS
Hydrazide and hydroxylamine	(Carboxymethyl)trimethylammonium chloride hydrazide (Girard T reagent, [4, 56]); 2-hydrazinopyridine [57]; *p*-toluenesulfonhydrazide [36] Girard P reagent [58] Hydroxylamine [25]	Ketolic C=O	LC-ESI$^+$/MS/MS MALDI/MS/MS LC-ESI$^+$/MS/MS
Benzyl bromide	Pentafluorobenzyl bromide [55, 59] 4-Nitrobenzyl bromide [60]	Phenolic OH	LC-APCI$^-$/MS/MS
Fluorobenzene and fluoropyridine	2,4-Dinitro-5-fluorobenzene analogues [54] 2-Fluoro-1-methyl-2-pyridinium *p*-toluenesulfonate [61]	Phenolic OH	LC-ESI$^+$/MS/MS
Sulfonyl chloride	Dansyl chloride, 1,2 -dimethylimidazole-4-chloride, pyridine-3-sulfonyl chloride 4-(1-H-pyrazol-1-yl)benzenesulfonyl chloride [62] 10-Ethyl-acridine-2-sulfonyl chloride [63]	Phenolic OH	LC-ESI$^+$/MS/MS LC-APCI$^+$/MS/MS
Carboxylic acid *N*-hydroxysuccinimide ester	*N*-Methyl-nicotinic acid *N*-hydroxysuccinimide ester [21]	Phenolic OH	LC-ESI$^+$/MS/MS
Carbonyl chloride or acetic anhydride	Picolinoyl chloride [64] 4-Nitrobenzoyl chloride [60] Pentafluorobenzoyl chloride [65] Acetic anhydride [66]	Both phenolic OH and alcoholic OH	LC-ESI$^+$/MS/MS LC-APCI$^-$/MS/MS GC-EI$^+$/MS LC-APCI$^+$/MS^2
o-Phenylenediamine	*o*-Phenylenediamine [67]	Estrogen *o*-quinones	LC-ESI$^+$/MS/MS

4. Sulfonyl chloride, e.g., dansyl chloride, 1,2-dimethylimidazole-4-chloride, and pyridine-3-sulfonyl chloride; 4-(1-H-pyrazol-1-yl)benzenesulfonyl chloride [62]
5. Carboxylic acid *N*-hydroxysuccinimide ester, e.g., *N*-methyl-nicotinic acid *N*-hydroxysuccinimide ester [21]
6. Carbonyl chloride and acetic anhydride, e.g., picolinoyl chloride [64]
7. *o*-Phenylenediamine [67]

The first class of derivatization reagents, hydrazide reagents and hydroxylamine, react with most of the ketolic steroid hormones and metabolites (ketone group on C_3, C_{17}, or C_{20}), i.e., androgens, progestagens, corticoids, and ketolic estrogens [4, 25, 36, 56]. However, they are not suitable for steroid hormones without ketolic group(s), such as estradiol, estriol and their related metabolites. Hydroxylamine is a typical derivatization reagent for steroid hormone profiling, because it can react with all the ketolic hormones, and provide unique mass fragments for each steroid moiety during LC-MS/MS analysis. When these unique mass fragments or daughter ions are used for multiple reaction monitoring (MRM) quantitation, the method selectivity is much higher than those using a daughter ion from a derivatization reagent, e.g., *m/z* 171 for dansyl ion [25].

The second to seventh classes of reagents react with hydroxyl steroid hormones and metabolites, especially estrogens. Pentafluorobenzyl bromide estrogen derivatives, belonging to the second class, are sensitive to both ESI^+ [1] and $APCI^-$ [55, 61] modes, and usually have lower limits of quantitation (LOQ) values under $APCI^-$ mode than those of derivatives of dansyl chloride and 2-fluoro-1-methyl-pyridinium *p*-toluenesulfonate under ESI^+ mode, because there were little interference from analogue compounds and the matrix background under $APCI^-$ mode. Nevertheless, the derivatization reaction of estrogens with pentafluorobenzyl bromide was ten times longer than the derivatization reaction with dansyl chloride (30 min vs. 3 min at 60 °C) [61]. A study by Higashi et al. indicated that the derivatization reactions of estrogens with 4-nitrobenzyl bromide (the second class), 2,4-dinitrofluorobenzene (the third class) and 4-nitrobenzoyl chloride (the sixth class) were not as complete as the reaction with 4-nitrobenzene sulfonyl chloride (the fourth class) [60].

The fifth class, carboxylic acid *N*-hydroxysuccinimide ester, is also not as reactive as sulfonyl chloride, and its derivative is not as sensitive to LC-MS/MS either [21]. The sixth class of reagents, carbonyl chloride and carboxylic acid anhydride, can react with both phenolic and alcoholic hydroxyl groups of steroids. However, the selectivity, speed, and completeness of derivatization reactions of these reagents with steroids are not as good as those of sulfonyl chloride derivatization reagents [60, 64, 66]. Since the other four classes of derivatization reagents, sulfonyl chloride, benzyl bromide, carboxylic acid *N*-hydroxysuccinimide ester, and fluorobenzene, are able to selectively react with phenolic hydroxyl group of estrogens and metabolites, the carbonyl chloride and carboxylic acid anhydride reagents become less preferable for derivatizing estrogens and metabolites. The seventh class, *o*-phenylenediamine, is a specific derivatization reagent for estrogen *o*-quinones, potential carcinogens, such as estrone-2,3-quinone, estrone-3,4-quinone, estradiol-2,3-quinone, estradiol-3,4-quinone [67].

Works published so far suggest that sulfonyl chloride is a preferred reagent for derivatizing estrogens and their metabolites, due to its reaction completeness and selectivity. In addition, a sulfonyl chloride reagent containing a basic or preionized nitrogen atom, e.g., on dansyl, pyridine, imidazole, pyrazole, or piperizine ring, could significantly enhance the ionization of estrogen derivatives under ESI^+ mode, and increase the detection sensitivity [4, 54, 62]. Dansyl chloride is a typical sulfonyl

chloride reagent used for derivatizing estrogens and metabolites from varieties of matrices, such as river water [50, 61], mouse plasma and brain [27], human urine [28], breast tissue [26], and serum [8]. However, dansyl derivatives have a disadvantage that the common dansyl fragment *m/z* 171 from background of the derivatization reagent and from those derivatives may have negative impacts on the method selectivity and accuracy during LC-MS/MS analysis, especially for those isomers with the same molecular ions and fragments, because the elevated dansyl fragment background noise may reduce the analyte signal–noise ratio, and cross interfere quantitation of other analytes with the same fragments [8, 25, 28].

3 Comparison of LC-MS/MS, GC-MS, and Immunoassays

3.1 LC-MS/MS and UHPLC-MS/MS vs. GC-MS and GC-MS/MS

The bioanalytical methods developed in recent years focused more on LC-MS/MS and GC-MS/MS techniques, because the earlier studies demonstrated that LC-MS/MS and GC-MS/MS are significantly more sensitive in analyzing estrogens and metabolites than LC-MS and GC-MS [18, 68]. Analyzing both unconjugated and conjugated steroid hormones directly is the major advantage of LC-MS and LC-MS/MS over GC-MS and GC-MS/MS, because the sample preparation procedures of deconjugation and derivatization can be avoided [5, 7, 40–42, 44]. In complex samples, separation of steroid hormones and metabolites by LC or GC is still one of the major concerns, because many steroid hormones and metabolites have the same molecular weights and MS fragments, and they may interfere with each other if not separated. For example, 2-hydroxyestrone and 4-hydroxyestrone have same molecular weight, even after they are derivatized with MSTFA for GC-MS analysis [48] or with dansyl chloride or *p*-toluenesulfonhydrazide for LC-MS/MS analysis [8, 35]. If the derivatives are not separated, GC-MS or LC-MS/MS is unable to distinguish the derivatives of 2-hydroxyestrone from those of 4-hydroxyestrone, whether the silylated, dansylated, or hydrozone steroid fragments are used for quantitation.

An LC column with a length of 150 mm can separate up to 23 steroid hormones and metabolites [20]. A typical LC-MS/MS method developed by Xu et al. was able to separate 15 estrogens and metabolites using a 150×2 mm, 4 μm LC column, which had a run time of 100 min [8, 28]. The separation efficiency can be improved by using smaller particle size LC columns, e.g., 100×2.0 mm, 2.5 μm column [69] or 50×2.1 mm, 1.8 μm column [21], also leading to a significantly reduced run time (e.g., less than 30 min). Similarly, ultra high performance liquid chromatography (UHPLC) is able to significantly improve separation efficiency and to reduce run time [40, 70–72]. Two-dimensional (2D) LC-MS/MS with column-switching technique has been used for determination of unconjugated and conjugated estrogens in

river water [51] and sediment [73], and this technique can significantly reduce the analysis time, and increase method separation capability and detection sensitivity. When estrogens and their derivatives of dansyl chloride and pentafluorobenzyl bromide are analyzed by HPLC, 2D-LC and UHPLC with ESI, APCI, APPI, and APCI/APPI MS modes, the UHPLC-ESI^--MS/MS significantly enhances sensitivities of the derivatives over the native estrogens, in the order UHPLC > 2D-LC > LC, and ESI > APPI > APCI = APCI/APPI [74]. On the other hand, the cleaner and more efficient supercritical fluid chromatography (SFC)-mass spectrometry was also used for analysis of estrogens and metabolites [75], but the SFC technology was less versatile and robust than LC, UHPLC, and GC.

Since the neutral molecules of steroid hormones and metabolites are not easily ionized under either $APCI^{+/-}$ or $ESI^{+/-}$ modes, LC-MS/MS is less sensitive when used directly in either $APCI^{+/-}$ or $ESI^{+/-}$ modes, with LOQs at ng/mL level as shown in Table 4 [20, 21, 52]. It has been observed that estrone, 16α-hydroxyestrone, 2-methoxyestrone, 4-methoxyestrone, and 2-hydroxy-3-methoxyestrone are sensitive to $APCI^+$ mode, while 2-hydroxyestrone and 4-hydroxyestrone are sensitive to $APCI^-$ mode, and even more sensitive to ESI^- mode [29]. Estrone, estradiol, estradiol, and estriol are sensitive to ESI^- mode, and testosterone is sensitive to ESI^+ mode [76, 77]. Similarly, estrone and estradiol are sensitive to $APPI^-$ mode, and testosterone is sensitive to $APPI^+$ mode with LOQs in a range of 1.5–10 pg/mL [22], which are comparable with those steroid hormones and metabolites derivatized with hydroxylamine or dansyl chloride, and detected under ESI^+ mode [2, 8].

As most steroid hormones and metabolites are already identified, high resolution MS technologies with higher selectivity, e.g., time of flight mass spectrometry (TOF-MS), Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), Orbitrap-MS, MALDI, etc., may not have major advantages over triple quadrupole MS in quantitative analysis, because the triple quadrupole MS takes shorter data acquisition time, leading to a higher sensitivity. For example, LC-ESI^--MS/MS is 4–6 times more sensitive than LC-ESI^--TOF-MS in analysis of estrogens [76]. On the other hand, MS interface also plays an important role in steroid hormone analysis. When analyzed by nanospray ES-Q-TOF-MS, Girard P derivative of testosterone at 0.5 pg/μg (0.1 pg of sample) can be detected, while 10 pg of sample is needed for matrix-assisted laser desorption ionization (MALDI)-Q-TOF-MS analysis, because of significant background interferences from the MALDI matrix [58].

GC-MS is a matured technology in analyses of steroid hormones, because GC-MS interface and electron impact ionization (EI) MS mode are stable and easily standardized [1]. GC-MS is a very powerful tool for profiling steroid hormones in biological matrices, such as that more than 70 steroid hormones and metabolites can be separated and quantitated by a single GC-MS run, with LOQ in range of 0.1–10 ng/mL [6, 48]. The derivatization procedure may not be a challenge, because there are many derivatization reagents available, e.g., BSTFA and MSTFA, and the silylation reaction is straightforward and quantitative. In addition, the sensitivity of GC-MS can be improved from low ng/mL to 0.6 pg/mL LOQ level by GC-MS/MS technology [23]. That is why GC-MS is still broadly used today for analyses of

Table 4 Comparison of GC-MS, GC-MS/MS, and LC-MS-MS in separation and sensitivity

			Method sensitivity, LOQ (S/N ≥ 5)								
			GC/MS	GC/NCI/ MS/MS	LC/APCI$^+$/ MS/MS	LC/APCI$^-$/ MS/MS	LC/ESI$^-$/ MS/MS	LC/ESI$^+$/ MS/MS	LC/ESI$^+$/ MS/MS	LC/APPI$^+$/ MS/MS	LC/APPI$^-$/ MS/MS
			Urine, 2 mL	Serum, 1.0 mL	Prostatic tissue	Serum, 0.5 mL	Milk, 10 mL	Urine, 0.2 mL	Serum	Serum, 0.2 mL	Serum, 0.2 mL
Compound	Abbreviation	FW	MSTFA derivatives (ng/mL)	PFB, MSTFA (pg/mL)	carbonyl derivative (ng/g) (LOD)	Underivatized (ng/L) (LOD)	Underivatized (pg/L)	Underivatized (ng/mL)	NH_2OH derivative (pg/mL)	Underivatized (pg/mL)	Underivatized (pg/mL)
Androgens											
Androstenedione	A-dione	286	2.0								
Dehydroepiandrosterone	DHEA	288	50.0					1			
Androstanedione	5α-dione	288	5.0								
Epitestosterone	Epi-T	288	0.1					0.3	10		
Testosterone	T	288	0.1					0.3		10	
5β-Dihydrotestosterone	5β-DHT	290	0.2							50*	
Androsterone	An	290	0.5					1			
Etiocholanolone	Etio	290	0.5					1			
Epietiocholanolone	Epi-Etio	290						1			
Epiandrosterone	Epi-An	290	5.0					1			
Androstenediol	A-diol	290	2.0		<0.35						
5α-Dihydrotestosterone	5αDHT	290	0.2					0.5		50*	
5β-Androstan-3α, 17α-diol	βαα-diol	292	1.0							200	
5β-Androstan-3β, 17α-diol	ββα-diol	292	0.5							200	
5α-Androstan-3α, 17α-diol	ααα-diol	292									

5β-Androstan-3β, 17β-diol	βββ-diol	292							
5α-Androstan-3α, 17β-diol	ααβ-diol	292	1.0				0.5		
5β-Androstan-3α, 17β-diol	βαβ-diol	292	0.5						
5α-Androstan-3β, 17α-diol	αβα-diol	292							
Epidihydrotestosterone	Epi-DHT	292	0.2						
5α-Androstan-3β, 17β-diol	αββ-diol	292	20.0						
5α-Androstan-3, 17-dione	5α-Adione	288					1		
16α-Hydroxyandros-tenedione	16α-OH-Adione	302							
11-Keto-androsterone	11-keto-An	304	0.5						
11-Keto-etiocholanolone	11-keto-Etio	304	0.5				0.5		
16α-Hydroxy-DHEA	16α-OH-DHEA	304	0.2						
11β-Hydroxyandroster-one	11β-OH-An	306	0.5						
11β-Hydroxyetiocho-lanolone	11β-OH-Etio	306	0.5				1		
Testosterone-17β-glucuronide	T-Gluc	464					0.3		
Estrogens									
Estrone	E1	270	0.2		37,000	20	1	<0.5 mL>	1.5
17α-Estradiol	17α-E2	272	0.1					1 <8>	
17β-Estradiol	17β-E2	272	0.1	0.6	143	50	0.3	1 <8>	2.5

(continued)

Table 4 (continued)

			Method sensitivity, LOQ (S/N ≥ 5)								
			GC/MS	GC/NCI/ MS/MS	LC/APCI$^+$/ MS/MS	LC/APCI$^-$/ MS/MS	LC/ESI$^-$/ MS/MS	LC/ESI$^+$/ MS/MS	LC/ESI$^+$/ MS/MS	LC/APPI$^+$/ MS/MS	LC/APPI$^-$/ MS/MS
			Urine, 2 mL	Serum, 1.0 mL	Prostatic tissue	Serum, 0.5 mL	Milk, 10 mL	Urine, 0.2 mL	Serum	Serum, 0.2 mL	Serum, 0.2 mL
Compound	Abbreviation	FW	MSTFA derivatives (ng/mL)	PFB, MSTFA (pg/mL)	carbonyl derivative (ng/g) (LOD)	Underiva-tized (ng/L) (LOD)	Underiva-tized (pg/L)	Underiva-tized (ng/mL)	NH_2OH derivative (pg/mL)	Underiva-tized (pg/mL)	Underiva-tized (pg/mL)
2-Hydroxyestrone	2-OH-E1	286	0.2			14			<8>		
4-Hydroxyestrone	4-OH-E1	286	0.2			50			<8>		
16-Keto-17β-estradiol	16-keto-E2	286	0.2			4,200			<8>		
16α-Hydroxyestrone	16α-OH-E1	286	0.2			4,800			<8>		
2-Hydroxy-17β-estradiol	2-OH-E2	288	0.2			14			<8>		
4-Hydroxy-17β-estradiol	4-OH-E2	288	0.2			30					
17-Epiestriol	17-epi-E3	288	1.0						<8>		
Estriol	E3	288	0.5			15,700	50	3	1 (8)		
16-Epiestriol	16-epi-E3	288	1.0			6,600			<8>		
4-Methoxyestrone	4-MeO-E1	300				8,700			<8>		
4-Methoxy-17β-estradiol	4-MeO-E2	302	0.5			19,200			<8>		
2-Methoxyestrone	2-MeO-E1	300	0.1			32,400			<8>		
3-Methoxyestrone	3-MeO-E1	300							<8>		
2-Methoxy-17β-estradiol	2-MeO-E2	302	0.1			61,500			<8>		
2-Hydroxyestriol	2-OH-E3	304	0.5								
Progestagens											
Progesterone	Prog	314	1.0					0.3	50		
5α-Dihydroprogesterone	5α-DHP	316	0.5								
5β-Dihydroprogesterone	5β-DHP	316	0.2								

20α-Hydroprogesterone	20α-DHP	316	0.5			
Pregnenolone	Preg	316	2.0			
Pregnanolone	P-one	318	2.0			
Allopregnanolone	Allo-P-one	318	0.5			
Epipregnanolone	Epi-P-one	318	2.0			
Isopregnanolone	Iso-P-one	318				
Pregnanediol	P-diol	320	1.0		0.3	50
17α-Hydroxyproges-terone	17α-OH-Prog	330	0.5			
11β-Hydroxyproges-terone	11β-OH-Prog	330	5.0			250
17α-Hydroxypregne-nolone	17α-OH-Preg	332	10.0			
21-Hydroxypregnenolone	21-OH-Preg	332				
Pregnanetriol	P-triol	336	0.5			
Pregnanediol glucuronids	P-diol-Gluc	496			0.5	
Corticoids						
11-Deoxycorticosterone	11-deoxyB	330				
5β-Dihydrodeoxycor-ticosterone	5βDHDOC	332				
Tetrahydrodeoxycor-ticosterone	THDOC	334				
11-Dehydrocorticos-terone	11-dehyd-roB	344				
11-Deoxycortisol	11-deoxyF	346				50
21-Deoxycortisol	21-deoxyF	346				
Corticosterone B		346				
Allodihydrocor-ticosterone	Allo-DHB	348				

(continued)

Table 4 (continued)

			Method sensitivity, LOQ (S/N ≥ 5)								
			GC/MS	GC/NCI/ MS/MS	LC/APCI$^+$/ MS/MS	LC/APCI$^-$/ MS/MS	LC/ESI$^-$/ MS/MS	LC/ESI$^+$/ MS/MS	LC/ESI$^+$/ MS/MS	LC/APPI$^+$/ MS/MS	LC/APPI$^-$/ MS/MS
			Urine, 2 mL	Serum, 1.0 mL	Prostatic tissue	Serum, 0.5 mL	Milk, 10 mL	Urine, 0.2 mL	Serum	Serum, 0.2 mL	Serum, 0.2 mL
Compound	Abbreviation	FW	MSTFA derivatives (ng/mL)	PFB, MSTFA (pg/mL)	carbonyl derivative (ng/g) (LOD)	Underiva-tized (ng/L) (LOD)	Underiva-tized (pg/L)	Underiva-tized (ng/mL)	NH_2OH derivative (pg/mL)	Underiva-tized (pg/mL)	Underiva-tized (pg/mL)
Tetrahydrocorticosterone	THB	350									
Tetrahydrodeoxycortisol	THS	350							5,000		
Cortisone E		360							5,000		
Cortisol F		362						0.3			
20α-Dihydrocortisone	20α-DHE	362									
20α-Dihydrocortisol	20α-DHF	364									
Allodihydrocortisol	Allo-DHF	364									
Tetrahydrocortisone	THE	364									
Tetra-11-dehydrocor-ticosterone	THA	364									
α-Cortolone		366									
β-Cortolone		366									
Tetrahydrocortisol	THF	366						1			
Allotetrahydrocortisol	Allo-THF	366						3			
α-Cortol		368						3			
β-Cortol		368									
Sterols											
Cholestenone		384									
Desmolesterol		384									

Cholesterol	Chol	386	2.0							
20α-Hydroxycholesterol	20α-OH-Chol	402								
24S-Hydroxycholesterol	24S-OH-Chol	402	2.0							
Lanosterol		428								
Reference			[6]	[23]	[16]	[21]	[52]	[20]	[2]	[22]

* <8> is 8 pg/mL in serum and derivatized with densyl chloride [8].

steroid hormones in human plasma [32], urine [7, 37, 39], and environmental water [34, 45, 78, 79]. If both LC-MS/MS and GC-MS/MS methodologies require similar sample preparation procedures, e.g., deconjugation, extraction, and derivatization, and they provide the same or comparable sensitivity in steroid hormone analyses, the choice of an LC-MS/MS or a GC-MS/MS methodology may depend on the instrument availability and the cost of the testing.

3.2 LC-MS/MS and GC-MS vs. Immunoassays

IA and RIA are broadly used technologies in steroid hormone analyses by both clinical laboratories and environmental agencies, because they are sensitive, convenient, simple, rapid, and inexpensive. Their disadvantage is lack of selectivity or specificity [4, 14, 15]. Since selectivity or specificity of bioanalytical methodologies is essential for clinical diagnosis testing, a number of studies have been conducted to compare LC-MS/MS and GC-MS methodologies with immunoassays in steroid hormone analysis. A study for determining nine androgens and three estrogens in blood showed that LC-MS/MS, GC-MS, and RIA provided similar results, however, RIA resulted in significant higher values for all related hormones than LC-MS/MS and GC-MS, due to its cross interferences from analogue compounds [80]. Another study also indicated that direct RIA overestimated estrogen sulfate in plasma than GC-MS and LC-MS/MS [65]. Similar results were observed in analysis of estrogens by IA and RIA against LC-MS/MS as well [19]. All these studies suggest that LC-MS/MS and GC-MS are more selective or specific and accurate than RIA. Nevertheless, LC-MS/MS and GC-MS instruments require more sophisticated expertise to perform the bioanalytical testing, and to provide reliable interpretations of the results for clinical diagnoses.

4 Standardization of Analytical Procedures

Development of LC-MS/MS or GC-MS analytical methods is just the first step in bioanalysis. A number of additional procedures should also be established to make the whole bioanalytical platform including reference standards, methodologies and data systems in compliance with scientific and regulatory guidelines or requirements. Otherwise, the results from various techniques, e.g., IA, RIA, LC-MS/MS, and GC-MS, may not be comparable and acceptable for clinical diagnosis. As there are a large number of scientific publications and clinical applications on steroid hormone analyses, organizations like the Center for Disease Control and Prevention, Division of Laboratory Science of National Center for Environmental Health have attempted to standardize the clinical laboratory practice, analytical methodology and data management in steroid hormone analyses (CDC/NCEH/DLS, [81–83]). The National Institute of Standards and Technology (NIST) has made efforts on

development of standard methods and reference materials for the determination of hormones in human serum [84–86]. A number of researchers also proposed a series of standardization procedures, including method development, validation, data interpretation and correlation between different bioanalytical techniques, and establishing international databases of human hormones [2, 14, 18]. In contrast to the clinical laboratories, EPA considers steroid hormones as environmental pollutants in water and sediments, and it has established an LC-MS/MS Method #539 following EPA Chemical QC Guidelines [77].

4.1 Guidelines for GLP, Reference Standards, and Analytical Methodology

Current Good Laboratory Practice from Food and Drug Administration (FDA GLP, 21CFR58) is a general guideline for pharmaceutical industry, and Good Laboratory Practice from Environmental Protection Agency (EPA GLP, 40CFR792) is a guideline for agriculture chemical industry, and environmental protection agencies and organizations. Both guidelines emphasize on compliance in personnel, facilities, articles, and documentation for animal and analytical experiments. In order to standardize the analytical methodology of steroid hormone analysis, the clinical and the environmental laboratories and facilities should follow FDA or EPA GLP guidelines. These guidelines include the following major requirements: (1) to train related analysts, (2) to calibrate and maintain instruments, (3) to characterize reference standard, (4) to conduct analytical testing following standard operation procedures (SOPs), (5) to record analytical procedures, protocols, reports, deviations, investigation, etc. Besides GLPs, guidelines for reference standards and analytical methodology are also important for steroid hormone analyses, as summarized in Table 5.

4.2 Reference Standards

The primary reference standards, including internal standards, of steroid hormones used for LC-MS/MS or GC-MS analyses in GLP laboratories should be obtained from authentic sources, e.g., US Pharmacopeia (USP) and NIST. Relative inexpensive secondary or working standards for daily testing may be used as alternatives of the primary standards, and they may be obtained from commercial sources. These working standards should be characterized against the primary reference standards using compendia or validated methods before being applied for GLP testing purposes. A reference standard program should be established in each laboratory, institute, or company to monitor the specification, quality, characterization, stability, storage, inventory, and replacement of those reference standards. A certificate of analysis should be issued after characterization of each standard batch, and it should include the information of manufacturer or source, date of manufacture, date of

Table 5 Guidelines for cGLP, reference standards, and analytical methodology

Guideline	Major topics
FDA GLP (21CFR58)	Ensures the quality and integrity of test data, personnel, facilities, articles, and documentation in pharmaceutical industry
EPA GLP (40CFR792)	Ensures the quality and integrity of test data, personnel, facilities, articles, and documentation in agriculture chemical industry and environmental protection organizations
USP34-NF29 S1 <11>	*General Requirements*/<11>USP Reference Standards—definition and applications
ISO guides for reference materials	ISO Guide 30:1992/Amd 1:2008 Revision of definitions for reference material and certified reference material ISO Guide 31:2000 Reference materials—Contents of certificates and labels ISO Guide 32:1997 Calibration in analytical chemistry and use of certified reference materials ISO Guide 33:2000 Uses of certified reference materials ISO Guide 34:2009 General requirements for the competence of reference material producers ISO Guide 35:2006 Reference materials—General and statistical principles for certification
USP34-NF29 S1 <1225>	Validation of compendial procedures
ICH Q2(1)	Validation of analytical procedures
IUPAC method validation	International Union of Pure and Applied Chemistry, Harmonized guidelines for single laboratory validation of method of analysis
NIST Tech Note 1297	Guidelines for evaluating and expressing the uncertainty of NIST measurement results
ISO 15193—Bioanalytical procedures	ISO 15193:2009 In vitro diagnostic medical devices—Measurement of quantities in samples of biological origin—Requirements for content and presentation of reference measurement procedures
ISO 5725—Accuracy (trueness and precision) of measurement methods and results	ISO 5725-1:1994 General principles and definitions ISO 5725-2:1994 Basic method for the determination of repeatability and reproducibility of a standard measurement method ISO 5725-3:1994 Intermediate measures of the precision of a standard measurement method ISO 5725-4:1994 Basic methods for the determination of the trueness of a standard measurement method ISO 5725-5:1998 Alternative methods for the determination of the precision of a standard measurement method ISO 5725-6:1994 Use in practice of accuracy values
FDA bioanalytical method validation	Guidance for Industry, Bioanalytical Method Validation
NIST methods and Reference Materials	Development of reference methods and reference materials for the determination of hormones in human serum
EPA	EPA Chemical QC Guidelines EPA method 539: Determination of hormones in drinking water by solid phase extraction (SPE) and liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) [77]

analysis, testing results (purity or strength, moisture, impurities, etc.), storage conditions, and expiration or retesting date. More detailed information on reference standard can be found in USP34-NF29 and International Organization for Standardization (ISO) Guides 30-35.

4.3 Standardization of Methodology

In general, the analytical methodology evaluates and defines bioanalytical/analytical procedures using a number of parameters, including selectivity or specificity, accuracy, precision, matrix effect, recovery, calibration model (linearity) and range, sensitivity (limit of quantitation—LOQ, and limit of detection—LOD), sample stability, ruggedness and robustness [87]. The USP and ICH guidelines listed in Table 4 focus on the role of analytical methodology in quality control and compliance of drug substances and drug products in pharmaceutical industry. The IUPAC, ISO, and NIST 1297 guidelines emphasize on the definition and evaluation of trueness and uncertainty in analytical methodology. The FDA guideline is the most direct guidance on bioanalysis for human and nonhuman (animal and biological) studies in pharmacology, toxicology, pharmacokinetics, and drug metabolism. Typical bioanalytical methodology guidelines and LC-MS/MS methods for steroid hormone analyses are shown in Table 6.

To standardize the steroid hormone analyses, standard operation procedures (SOPs) should be generated for all laboratory functions, e.g., facilities, instrument, reference standards, samples, procedures, data collection and processing, documentation, etc., following GLP, reference standard, and the methodology guidelines. All the laboratory activities should follow these SOPs. Furthermore, the experimental procedures or techniques, e.g., deconjugation, extraction (LLE or SPE), derivatization, isotope dilution, instrument setting (e.g., LC-MS/MS modes), etc., should also be standardized. It is a challenge to ask different laboratories meet the same criteria, e.g., sensitivity and precision, using different models of instruments, and to make the results comparable and reliable for clinical diagnosis, due to the significant differences in LC-MS/MS hardware (configuration, ionization modes, and parameter setting) and software (data acquisition speed and processing) from different vendors and different models.

Standardization of GC-MS methodology for steroid hormones is relatively straightforward, because most of the unconjugated steroid hormones have a hydroxyl group(s) at C_3, C_{11}, C_{17}, or C_{21} position, which may be easily derivatized with a trimethylsilylation agent, e.g., MSTFA, and derivatives may be well separated by GC, and detected by MS using EI^+ mode [6]. Similarly, standardization of LC-MS/MS methodology for estrogens and metabolites is also not very complicated, because all unconjugated estrogens and metabolites have phenolic hydroxyl group(s) at C_3, and C_2 or C_4 positions, which may be derivatized with a sulfonyl chloride, e.g., dansyl chloride, and analyzed LC-MS/MS using ESI^+ [8, 84, 88]. The other derivatization agents, e.g., hydrazide and hydroxylamine, may react only with those ketolic

Table 6 Typical bioanalytical methodology guidelines and LC-MS/MS methods for steroid hormone analyses

Parameter	FDA Guidance for bioanalytical method validation (http://fda.gov/edcr/guidance/index.htm)	EPA Method 539—7 hormones in drinking water by LC/ESI$^{+/-}$-MS/MS (http://water.epa.gov/drink/)	LC/ESI^{+}-MS/MS methods for hormones in serum following NIST 297 and ISO15193 guidelines [84, 85]	An LC/ESI^{+}-MS/MS method for estrogens in plasma following IUPAC guideline [88]
Specificity	No interference from biological matrix endogenous compounds and metabolites	Interference from reagent blank ≤ 1/3MRL	Endogenous hormones were subtracted from the blank	>95 % certainty of no analyte in blank (n = 10)
Accuracy	Nominal ±15 % Nominal ±20 % at LLOQ	Nominal ±30 % at >2× MRL Nominal ±50 % at ≤2× MRL	100.7–101.8 % for estradiol 100.0–100.3 % for testosterone	93–105 %
Precision	%RSD ≤ 15 % at ≥ 3 levels n ≥ 5 at each level %RSD ≤ 20 % at LLOQ	%RSD ≤ 20 % n = 4–7	CV ≤ 2.2 % Uncertainty evaluation: Coefficient of variation (CV) 1 % Uncertainty of volumetric error 0.3 % Uncertainty of reference standard 0.1 % Uncertainty of weighing 0.5 % Uncertainty of other systematic errors Combined standard uncertainty Coverage factor Expanded uncertainty Relative expanded uncertainty (%)	Intraassay CV = 2–9 % Interassay CV = 4–12 %
Calibration	≥6 (6–8) standard points spiked in matrix including LLOQ A function of analyte vs. response within the calibration range	≥5 standard points spiked in 50%MeOH/reagent water The lowest at or below MRL Linear or quadratic regression	six points calibration curve	Seven points calibration curve
QC samples	Replicates at three levels, e.g., LQC (3× LOQ), MQC and HQC 67 % (4 out of 6) within ± 15 % of nominal value	Single run of LQC, MQC, … and HQC LQC within ± 50 % of nominal value, and others within ± 30 %	Single run of working standards at two levels	NA

Sensitivity	LLOQ = S/N ≥ 5 Accuracy: 80–120 %	MRL is confirmed by: Upper PIR = (Mean + HR_{PIR})/fortified concentration × 100 ≤ 150 % Lower PIR = (Mean − HR_{PIR})/ fortified concentration × 100 ≥ 50 %	LOD = S/N = 3	LOQ = the lowest measureable concentration of actual sample with interassay CV < 20 %
Sample stability	3× Freeze–thaw cycles for samples between −70 °C and room temperature; 4–24 h at room temperature; long-term storage; sample stock and postpreparation stability.	5–10 ng/L × 6 at ≤ 6 °C for 28 days	NA	3× Freeze–thaw cycles for samples between −20 °C and room temperature; Ambient and 4 °C for 7 days
Internal standard	Isotope labeled analyte with optimized binding assessment, and verified standard curve	IS peak area within ±50 % of each analyte $^{13}C_6$-Estradiol, 16α-Hydroxyestradiol-d_2, Testosterone-d_3, $^{13}C_2$-Etheylnylestradiol	IS peak area within ± 50 % of each analyte Testosterone-d_3, Estradiol-d_3	Estron-d_4, Estradiol-d_5
QC samples	Replicates at three levels, e.g., LQC (3× LOQ), MQC and HQC 67 % (4 out of 6) within ± 15 % of nominal value	Single run of LQC, MQC, … and HQC LQC within ± 50 % of nominal value, and others within ± 30 %	Single run of working standards at two levels	NA
Sensitivity	LLOQ = S/N ≥ 5 Accuracy: 80–120 % Precision: ≤20 %	MRL is confirmed by: Upper PIR = (Mean + HR_{PIR})/fortified concentration × 100 ≤ 150 % Lower PIR = (Mean − HR_{PIR})/ fortified concentration × 100 ≥ 50 % HR_{PIR} = 3.963 S	LOD = S/N = 3	LOQ = the lowest measureable concentration of actual sample with interassay CV < 20 %

(continued)

Table 6 (continued)

Parameter	FDA Guidance for bioanalytical method validation (http://fda.gov/edcr/guidance/index.htm)	EPA Method 539—7 hormones in drinking water by LC/ESI$^{+/-}$-MS/MS (http://water.epa.gov/drink/)	LC/ESI^{+}-MS/MS methods for hormones in serum following NIST 297 and ISO15193 guidelines [84, 85]	An LC/ESI^{+}-MS/MS method for estrogens in plasma following IUPAC guideline [88]
Sample stability	3× Freeze–thaw cycles for samples between −70 °C and room temperature; 4–24 h at room temperature; long-term storage; sample stock and postpreparation stability.	5–10 ng/L ×6 at ≤6 °C for 28 days	NA	3× Freeze–thaw cycles for samples between −20 °C and room temperature; Ambient and 4 °C for 7 days
Internal standard	Isotope labeled analyte with optimized binding assessment, and verified standard curve	IS peak area within ±50 % of each analyte $^{13}C_6$-Estradiol, 16α-Hydroxyestradiol-d_2, Testosterone-d_3, $^{13}C_2$-Etheylnylestradiol	IS peak area within ± 50 % of each analyte Testosterone-d_3, Estradiol-d_3	Estron-d_4, Estradiol-d_5
Sample preparation	Extraction recovery (at three levels) of analyte and internal standard should be consistent, precise, and reproducible.	SPE, without derivatization	SPE + LLE (≥80 %), no derivatization for testosterone, Estrogens derivatized with dansyl chloride	LLE (100 %), derivatized with dansyl chloride

steroids, but will miss those nonketolic steroids [4, 25, 56]. If steroid hormones are analyzed without derivatization, they may need to be analyzed by LC-MS/MS using both positive and negative modes for different hormones, and the LOQ values of steroid hormones fall in a very broad range, because of the sensitivity variations of steroid hormones to different MS modes [22, 29, 77].

5 Applications of Mass Spectrometry in Steroid Hormones Analyses

5.1 Clinical Chemistry and Food Safety

Varieties of LC-MS/MS and GC-MS technologies and methodologies have been developed and applied for clinical chemistry diagnostic testing. For example, adrenal steroids, glucocorticoids, androgens, and estrogens are biomarker of many endocrinology diseases [2], pediatric development syndromes [9], and congenital adrenal hyperplasia [89]. Endogenous steroids are also related to prevalent cardiovascular disease in old men and women [90], and other age-related diseases [60]. Elevated estrogens and metabolites in plasma and serum of postmenopausal women are used as biomarkers for risk assessment of breast, ovary, and thyroid cancers, bone homeosis and osteoporosis in postmenopausal women [4, 15, 39, 91]. Androgens may be related to prostate cancer progression and treatment [31], and hyperandrogenism may cause polycystic ovary syndrome and androgen-secreting tumors [32].

In addition, steroid hormones in many kinds of biological fluids and tissues have been determined by LC-MS/MS and GC-MS technologies and methodologies. For example, LC-MS/MS has been utilized for monitoring (1) plasma corticosteroids and metabolites to evaluate their therapeutic and side effects as clinically used medicines [92], (2) estrogens in human cerebrospinal fluid [93] and peritoneal fluid [94], (3) urinary endogenous estrogen metabolites [95, 96], (4) estrogens in breast tissue [26], and (5) steroid hormones as residuals in edible matrices [12]. Polyphenol phytoestrogens in foods and human biological fluids are also measured by mass spectrometry technologies [97].

5.2 Antidoping Steroid Screen for Athletes and Racing Horses

Corticosteroids are used by some athletes and racing horses to enhance their performances. In order to prohibit drug doping, many sport organizations, e.g., International Olympic Committee, attempt to monitor the corticosteroids in urine of athletes. An example of earlier GC-MS method in steroid screen consists of procedures of deconjugation of glucuronide and sulfate, derivatization with MSTFA and GC-MS analysis [98]. The more recent LC-MS/MS methods separate the corticosteroids by

LC, and analyze them with ESI-MS (either positive or negative mode) without derivatization [10, 99]. Similarly, corticosteroids in racing horse urine are monitored by LC-ESI-MS/MS as well [11].

5.3 Determination of Steroid Hormones as Environmental Pollutants

Steroid hormones are found as pollutants in drinking water, waste water, river and sediments. The major concerns of analytical methodologies for monitoring steroid hormones from environmental samples are extraction techniques from aqueous or solid matrices. Since sample volume or amount is not an issue in most cases, SPE is the method of choice. Both LC-MS/MS and GC-MS technologies are broadly applied for steroid analyses of environmental samples, such as LC-MS/MS analyses of steroid hormones in effluents of wastewater treatment plants [100] and estrogens in water [101, 102], and GC-MS analyses of steroid hormones in environmental water [34, 45, 78, 79]. A study by Grover and colleagues showed that GC-MS was the simplest technique in determination of steroid hormones in environmental water samples, but lack of sensitivity; LC-MS/MS was more sensitive than GC-MS, but susceptible to matrix interferences; and GC-MS/MS was the recommended technique, because it was more selective and sensitive than GC/MS and LC-MS/MS [103].

6 Summary

LC-MS/MS has similar sensitivities in analysis of steroid hormones as IA, RIA, and GC-MS/MS, but LC-MS/MS and GC-MS/MS have much higher selectivity or specificity. The steroid hormones from different sources or matrices should be extracted with LLE or SPE, depending on the sample volume and matrices. Isotope (as internal standard) dilution is a standard procedure for quantitative analysis of steroid hormone and metabolites. Deconjugation is required in order to determine the total steroid hormones, because the very low levels of unconjugated and conjugated steroid hormones in biomatrices may not be feasible to be analyzed at the same time by LC-MS/MS, GC-MS, or GC/MS/MS. Derivatization can enhance MS detection sensitivity for many steroid hormones and metabolites, while the derivatization reagents and procedures should be selected based on the techniques of LC-MS/MS, GC-MS, or GC/MS/MS, and their ionization modes. Depending on the objectives of the method applications, bioanalytical procedures should be developed and validated according to the scientific and regulatory guidelines, e.g., FDA, EPA, ISO, NIST, IUPAC, and then these procedures may be standardized following CDC/NCEH/DLS practices. LC-MS/MS is increasingly applied for steroid analysis in research, clinical, pharmaceutical, and food industries, sports and environmental testing, due to its selectivity, sensitivity, and versatility.

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