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Review

Measurement of Steroids in the Placenta, Maternal Serum, and Fetal Serum in Humans, Rats, and Mice: A Technical Note

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Abstract: Steroid hormones are vital for a successful pregnancy. The placenta is attached to the uterine wall and is the major organ of communication between the mother and the fetus through the umbilical cord and the transfer of compounds (including the production and actions of steroids) across the villous placenta. Therefore, a correct understanding and measurement of steroid levels across the maternal–placental–fetal interface is essential. We have experience spanning more than two decades and have published more than 40 papers using a variety of methods to assess circulating and placental steroid levels. In this review, we discuss various methods for steroid detection and quantitation, as well as their advantages and disadvantages. This document provides technical guidance for best practices that, in our estimation, can assist researchers in more easily and correctly performing these studies. Critical methodological considerations, including tissue collection, tissue processing, and analytical factors (sensitivity, selectivity, matrix effects, and internal standards), are covered. We highlight important differences between human and rodent tissues as they relate to steroid levels in pregnancy and the interpretation of results, and provide guidance for best practices in future studies.

Keywords: analytical detection; ELISA; mass spectrometry; reproduction; progesterone; estradiol; stress; LC-MS/MS

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1. Introduction

Steroid hormones are vital to the establishment and maintenance of pregnancy and are involved in producing and coordinating the multitude of adaptations required for a successful pregnancy to occur [1]. While steroidogenesis is one of the major functions of the human placenta, in all species, a coordination between maternal, placental, and fetal steroid production is required to produce the range and concentration of steroid hormones required [2]. Alterations in steroidogenesis or steroid metabolism can lead to a number of pregnancy complications and pregnancy loss [3]. Therefore, evaluating steroid profiles in pregnancy can help us understand the pathophysiology of a wide range of clinical pregnancy complications. The use of treatments that affect steroid levels and metabolism may seem promising but should be used with caution as compounds disrupting steroidogenesis can have detrimental effects. This is highlighted by the use of diethystilbestrol (DES), a synthetic estrogen that was used to maintain pregnancy, but caused reproductive abnormalities arising in the teenaged offspring of mothers that ingested DES in pregnancy.

The consequences of this are still being studied in the grandchildren of women exposed to DES between 1950 and 1970 [4–6]. Further, studies of steroids extend beyond human reproduction, and include reproduction of coral, feminization of fish and frogs, and behavior of birds [7–11].

Although rodent models (e.g., laboratory rats and mice) have been essential in the advancement of reproductive studies, some important differences exist in steroid production and function between rodents and humans. In rodents, steroids are primarily produced by the ovaries with assistance from fetal tissues in the second half of pregnancy, but the corpus luteum must produce progesterone through the entirety of gestation [12,13]. Additionally, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) are not major circulating steroids in rats and mice, as they are in humans and other primates [14,15]. In rodents, steroid metabolism also occurs, as sulfotransferases (SULTs) and UDP glucuronosyltransferases (UGTs) are active in placenta and fetal liver. In humans, the placenta takes over progesterone production from the ovaries at 8 weeks of gestation; however, maternal and fetal contributions are needed [16]. The human placenta does not express the levels of cytochrome P450c17 (CYP17) necessary to transform cholesterol into adequate levels of estrogens [17]. Instead, DHEA and DHEAS are transported into the placenta from maternal and fetal circulation and converted to estrogens by 3β-HSD and aromatase. The human placenta expresses SULTs and UGTs, but the fetal liver lacks functional UGTs, affecting the steroid metabolome [18–21].

Additionally, several structural differences exist between rodent and human placentas. The human placenta is hemomonochorial, meaning that only one layer of cells (the trophoblast) separates the maternal blood from the fetal circulation [12]. In contrast, rat and mouse placentas are hemotrichorial, with three layers of cells (the decidua, trophoblast, and labyrinth zone) between maternal and fetal circulations [2]. It is of note then that both the human and rodent placentas have a decidua, but in human placentas (that do not invade the maternal uterus) the decidua is one cell-layer thick, as compared to rodent placentas that partially invade the uterus and the decidua is several cells thick [2,12,16]. Finally, the uteri themselves are different—in humans generally carrying a single fetus (more rarely, multiples), it is a pear-shaped organ [16], whilst rat and mouse uteri carry multiple fetuses in a horn-shaped organ. Hence, in addition to functional differences such as in steroid production, circulation, and clearance; these structural differences are usually taken into account when comparing rodent models to human reproduction.

A variety of methods are available for steroid quantification in tissues, and technological advancements in recent decades have increased specificity, sensitivity, and sample throughput for analysis. Immunoassays, both radioimmunoassay and enzyme-linked immunosorbent assay (ELISA), have traditionally been used to measure steroids in biological samples. These methods are available as commercial kits that are generally cost effective and easy to perform. However, immunoassays can present limitations in specificity (antibody cross-reactivity), sensitivity, or matrix effects. Liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS) is the current "gold standard" for steroid profiling [22]. Highly specific LC-MS or GC-MS overcomes the specificity limitations of immunoassays and presents the opportunity for simultaneous analysis of multiple steroids. The latter point is critical when samples are small (e.g., microdissected rodent placenta) or quantitation is low. However, MS is highly technical, requiring expertise in method development and instrument operation. Not all laboratories can afford MS instruments and, in addition, many laboratories lack access to trained MS technicians, in part because the training is difficult and time consuming. In contrast, basic plate reading spectrophotometers required for immunoassays are less expensive instruments. The operation of these spectrophotometers can also be learned to high technical proficiency in a relatively short period and does not require highly experienced operators/training.

Despite the generally high levels of steroids in pregnancy, the measurement of steroids in reproductive tissues presents certain challenges. Our laboratories, separately and in collaboration, have over two decades of experience in these areas, over 30 published

papers in steroid metabolism and detection in general, and several more in preparation. In order to assist others in the field, we present a technical note collecting our experiences of best practices. Briefly, sample collection and preparation can affect the steroid levels measured. For example, the heterogeneity of tissue must be considered in collection, as well as the ability of steroids to freely diffuse across cell membranes. In sample cleanup, adequate removal of compounds, such as lipids, that can cause assay interference must be performed. Finally, the differences in steroidogenesis and steroid metabolism across the species discussed above need to be acknowledged when using animal models. These issues are more comprehensively discussed below and solutions for past problems are presented.

2. Materials and Methodological Considerations

2.1. Tissue Collection and Processing

Human placenta samples: Informed consent and Institutional Review Board (IRB) approval.

The placenta is a valuable and unique organ for research as it is can be collected (with ethical approval and informed consent) immediately following birth. Additionally, because placental tissues are normally discarded after birth, they are regarded as medical waste and therefore, ethical approvals are easier to receive. However, research involving human tissues requires Institutional Review Board (IRB) approval. Additionally, pregnant people must provide written informed consent for the collection and inclusion of placental tissues for study. The potential for studying pregnancy outcomes as related to placental steroid hormones also requires accompanying clinical chart profiles of the pregnant patient and the neonate, which should be anonymized and deidentified. Immense coordination and time are required to prospectively collect tissues, and is a collective effort by physicians, nurses, and researchers. As such, reproductive biorepositories provide countless benefits for sourcing human tissue. These benefits include consistencies in the methods of collection, treatment, and storage of tissue, which should be considered in advance. The comparison of results between research groups requires clarity regarding collection and processing methods.

Non-human placenta samples: Institutional Animal Care and Use Committee (IACUC) approval.

IACUC approval is required for work with vertebrates, including rodents, sheep, pigs, etc. Such IACUC protocols must detail housing conditions, experimental procedures, and euthanasia. Euthanasia protocols often include deep anesthesia (e.g., via isoflurane) prior to euthanasia. If glucocorticoids, such as corticosterone and cortisol, are of interest, then timing is critical, and anesthesia should be rapid. In many studies, euthanasia is completed within 3 min of initial disturbance, to avoid acute increases in circulating glucocorticoids. In studies of the rodent placenta, tissue collection often occurs prior to birth, rather than after birth.

There were multiple studies contributing to this work that were covered by Institutional Biosafety Approvals from the University of British Columbia: (B22-0202 and B18-0009), Animal Care and Use Committee Approvals at the University of British Columbia (A19-0058 and A19-0227), as well as Clinical Research Ethics Board at the University of British Columbia (H14-00092) and The University of Hawaii IRB for Human Subjects (CHS 15080).

2.2. Sample Collection

Ideally, human placental tissue should be collected immediately after birth or a cesarian section, but certainly within two hours of delivery. Tissue should be stripped of decidua, the umbilical cord trimmed, and snap frozen in liquid nitrogen if desired. The human placenta weighs ~500 g at birth, and if sampling is to occur at the time of collection, location is important to consider. The human placenta is a heterogeneous organ; regional differences exist in terms of perfusion, cell type, and gene expression. Depending on the type of studies to be performed on the tissue, different sampling techniques may need to be

employed. However, there are no agreed upon standards for collection, sampling, and storage. A review of the challenges of taking a representative placental sample and strategies for sampling has been published by Mayhew [23]. Additionally, Burton et al. published an opinion piece discussing the challenges surrounding placental tissue collection, the importance of conditions for processing and storing tissues in a biobank, and proposed a protocol for optimum sample collection [24]. Below, we discuss the techniques we have used to prepare human and rodent placental tissue for steroid analysis.

Rodent placentas are collected by a cesarian section with the dams deeply anesthetized under isoflurane. Uteri are removed manually and opened with surgical scissors, placentas are separated from the uterus washed and preserved (snap frozen, RNA LaterTM, paraformaldehyde as required), and resorption sites are counted. Fetuses are euthanized immediately, and organs harvested as necessary. After the procedure, the dams are euthanized, and organs harvested as needed. Alternatively, dams may be deeply anesthetized with isoflurane within 2 min, decapitated, and blood collected. Fetuses and placentas are then counted and collected. In the specific case of measuring glucocorticoids, tissues must be collected within 3 min of the initial disturbance. For most reproductive tissues and steroids, collection is complete within 10 min of cesarian section or euthanasia.

2.3. Sample Processing

Prior to steroid analysis, the tissue sample must be processed into a homogenate such that steroids can be extracted. There are several methods for homogenizing tissue, including manually grinding tissue in a tube with a fitted pestle or Dounce homogenizer, an electric hand-held homogenizer such as the Tissue Tearor (BioSpec Products, Inc., Bartlesville, OK, USA), or a bead mill homogenizer. Our laboratories most often utilize an electric homogenizer or a bead mill homogenizer [18,19,25,26]. For processing larger amounts of tissue, such as human placental samples or whole rodent placenta, the hand-held homogenizer is routinely utilized. Briefly, tissue is thawed and wet weight is recorded, followed by homogenization in buffer (1:3 w/v) in a tube on wet ice. Lysate can be used fresh, processed further into S9 fraction, or aliquoted and frozen at −80 °C until future use. While this method produces a quality lysate that can be used for a variety of steroid extraction techniques, it can be time consuming when processing a large number of samples, as the homogenizer needs to be cleaned between each sample, and is less practical for small tissue amounts. The bead mill homogenizer is higher throughput, prevents cross contamination, and is more useful for small tissue amounts. To prepare tissue for steroid extraction, we have lysed samples directly in methanol and as well, several other solvents. This method can be used to homogenize many types of placental and other reproductive tissue samples, notably in our hands rat and human placental tissue weighing 5 mg or less [27–29], yielding consistent results for steroid measurements.

2.4. Analytical Considerations

Matrix Effects

Steroid measurements can be affected by steroidal and non-steroidal molecules present in the sample. These matrix effects can be caused by structurally similar steroids, such as steroid isomers and steroid metabolites, or by other lipophilic compounds [25]. In immunoassays, matrix effects can be produced by antibody cross-reactivity, while in mass spectrometry, ion suppression or enhancement effects are caused by co-eluting compounds that mainly affect steroid ionization efficiency. Matrix effects are assessed by using increasing amounts of sample and evaluating linearity and parallelism to a calibration curve in neat solution [11]. The process of sample cleanup, such as steroid extraction, helps to overcome matrix effects. When matrix effects are inevitable, the use of a matrix surrogate (e.g., charcoal-stripped plasma) in the calibration curves is often useful. In the case of steroids, the charcoal removes background levels of steroids from serum or other tissue samples, allowing for "clean" standard curves to be constructed in the correct matrix of interest, so that analytes can be clearly detected and quantitated free of interference. In

addition, the use of stable isotope internal standards can also account for matrix effects. Steroid measurement must be validated in each species for each sample type (e.g., placenta or plasma).

As larger tissue amounts increase the risk of matrix effects, minimizing tissue amounts is one way to overcome matrix effects, but this may not always be possible. In our LC-MS method that involved the direct homogenization of tissue in methanol prior to steroid extraction, matrix effects could be avoided by lowering the amount of tissue homogenized or further diluting the homogenate prior to extraction. Matrix effects were observed at placenta tissue amounts greater than 5 mg; however, the method was sensitive enough to accurately measure steroid concentrations in 1–2 mg of placenta tissue. In the event that matrix effects cannot be mitigated at the amount of tissue required for screening, one can create a surrogate blank matrix (described above). When using commercial ELISAs to measure steroids, assays must be performed as per manufacturer's instructions; thus, it is not always possible to mitigate matrix effects. Evaluating the recovery in spiked vs. unspiked samples can determine if absolute quantitation is possible in the tissue, or if only relative comparisons can be made.

The two major matrix effects [in technical terms] are ion expansion (also called ion enhancement) and ion suppression, the latter being most common in mass spectrometric detection. Ion suppression occurs when a component of the matrix interferes with analyte detection, either through making the extraction and detection of analytes less complete (such as proteins binding analytes so they cannot be extracted well) or through direct interference in ionization within the instrument. In both circumstances detection of analytes (such as steroids) may be compromised, usually through incorrect detection and quantitation (either too low [suppression] or too high [background contamination or ion expansion/enhancement]), leading to misleading analyte characterization. Due to the complex nature of the tissue homogenates and challenges with liquid-liquid extraction (LLE), and solid-phase extraction (SPE) [30–35] we have found that ion suppression is the more common situation [34] with placental and other reproductive tissue lysates. Conversely, ion expansion/enhancement is possible. In this case, either there are background levels of the same compound or there is a non-specific compound that separates in at the same time and ionizes with the same signature as the analyte of interest, causing detection and quantitation problems. We have observed this latter phenomenon from a non-specific compound only once in practice when trying to detect NSAIDs in umbilical cord lysates, necessitating the use of a different internal standard [36].

2.5. Steroid Extraction

There are three main methods for the extraction and cleanup of steroids for analysis: protein precipitation, LLE, or SPE. The best option depends on the sample type, amount of sample available, and the concentrations of steroids within the tissue. The extraction process can affect steroid measurement, and therefore recovery experiments are necessary for method validation. Recovery can be assessed by comparing a spiked neat solution with a known amount of steroid with both unspiked and spiked biological samples. The steroid concentration in the spiked matrices should be equal to the sum of the unspiked matrices and the spiked neat solution. Recoveries must be assessed for all the different types of matrices used.

Several protein precipitation techniques exist; however, the addition of an organic solvent (methanol or acetonitrile) is the most common for preparing biological samples for LC-MS/MS. Because proteins interfere with analyte detection, their removal is essential for good resolution. This can be achieved by mixing the homogenized tissue samples with approximately 5 times the volume of organic solvent, lowering the solubility of proteins, and causing them to precipitate out of solution, thereby preventing any interference with steroid (or other analyte) analysis. The precipitates are concentrated by centrifugation, and the supernatant is removed and used for analysis. While this method is quick, non-technical, and cost-effective, it can result in analyte loss due to protein binding and lower

sensitivity due to the presence of interfering compounds that are not removed. Accordingly, this method is suitable when there are larger sample volumes and steroid concentrations are higher. We have used this method for the extraction of steroid sulfates from human maternal plasma and fetal serum with good results (unpublished data). The circulating concentrations of these compounds are in the range of 500–2000 ng/mL for DHEAS and 100–500 ng/mL for estrone sulfate, far above the lower limit of quantitation (LLOQ) of our assays.

Liquid–liquid extraction with organic solvents is another common method of steroid isolation [23–25]. Steroids readily dissolve in organic solvents (e.g., methylene chloride, MTBE), and LLE provides another fast and cost-effective method for steroid extraction. However, other compounds, such as nonsteroid lipids, are also soluble in organic solvents and may disrupt the quantitation of steroids [26]. Liquid-liquid extraction of steroids is straightforward, fast, and more economical than SPE protocols (see below). It is applicable to small plasma samples and small quantities of tissue, for which LLE effectively removes matrix interference.

To isolate steroids from large amounts of tissue samples, SPE might be necessary. Here, a carbon 18 column is primed and equilibrated, and then samples are loaded onto the columns. Afterwards, samples can be washed to remove interfering substances, and then analytes of interest are eluted [28]. In contrast to liquid–liquid extraction, SPE allows the use of larger amounts of sample. Further, an additional separation of steroids can be performed with SPE [29]. Using increasing percentages of organic solvent, conjugated (i.e., sulfated, glucuronidated) and free steroids can be collected separately. On the other hand, SPE can increase steroid loss during the extraction, and column materials (e.g., plastics) can leach out and affect sensitive steroid assays. SPE requires C18 columns and larger amounts of organic solvents, which have economic and ecological costs. Further, the number of samples that can be processed at one time is limited by the equipment used to hold the SPE columns (e.g., 24-position vacuum manifold).

In the placenta, maternal plasma, and fetal serum, the levels of most steroid hormones are sufficiently high that quantitation can be performed using protein precipitation or LLE techniques. We have had success using both methods to quantify unconjugated steroids and steroid sulfates. In our experience, SPE was not needed to quantify steroids in human or rodent placenta, and samples could be acceptably purified and detected using LLE techniques, likely due to their high abundance.

2.6. Stripping Blanks

For certain methods, including measurement of steroid sulfates in plasma, the standard curve must be prepared in a steroid-free matrix. Steroid-free human serum can be purchased from commercial vendors or prepared in-house. Purchasing charcoal-stripped serum does not ensure it is completely free of steroids. In our experience, serum must be charcoal stripped three times in order to adequately remove steroids and steroid conjugates for the matrix to be considered blank. We have used a method of charcoal stripping adapted from Carter (1978) as follows: To aliquoted serum or plasma, 140 mg activated charcoal power is added per milliliter. Samples are vortex mixed and shaken for 8 h or overnight at 4 °C. Following incubation, tubes are centrifuged at 2000× *g* for 20 min, the supernatant is removed and spun again for 10 min at 5000× *g*. To the resulting supernatant, 83 mg of kaolin is added per 1 mL of starting serum. Samples are vortexed, and the kaolin gathers any residual charcoal. Following a further 10 min of centrifugation, supernatant is forced through a 0.22 µm syringe filter. This process, repeated 3× will remove over 99% of steroids. A caveat to this method is the amount of sample loss that occurs following successive rounds of charcoal stripping, necessitating the use of large volumes of starting matrix. This may not be an issue for stripping human serum or plasma, but for tissue lysate, this could be a problem when working with small tissue amounts such as rodent placenta, particularly if extra tissue is not available for comparison.

2.7. Internal Standards

Internal standards are used in mass spectrometry to account for interferences caused by the sample matrix, or losses during sample preparation. There are two commonly used internal standards, deuterated and 13carbon labeled stable isotopes. Deuterium-labeled internal standards are those in which several hydrogen atoms are replaced by deuterium and are often lower in price. However, under certain conditions (e.g., sample preparation protocols), they can lose some deuterium atoms, which will affect results [37]. Further, when using a high-resolution separation technique, such as gas chromatography or ultra-high performance liquid chromatography, the deuterated internal standard can have a small but noticeable shift in retention time relative to the analyte. These disadvantages can be overcome with the use of 13carbon-labelled internal standards that have closer physicochemical properties to their analytes and therefore behave more similarly during chromatography [30]. These 13carbon-labelled standards are reliable; indeed, they are considered the "gold standard", although they come at a higher cost, which is a consideration particularly relevant for academic researchers.

2.8. Derivatization

Derivatization of samples involves the chemical transformation of a compound of interest to improve analytical capabilities and outcomes. Reproductive tissues, particularly the human placenta, contain high levels of steroid hormones. In our experience, derivatization is not necessary to increase the sensitivity or selectivity of assays to measure steroids in placenta, primarily due to the abundance of the analytes. Using both positive and negative ionization modes in LC-MS methods, progestogens, androgens, and estrogens can be quantified in rodent and human placental tissue without derivatization (unpublished data), and we have also shown this in other tissues [10].

2.9. Selectivity vs. Sensitivity

Immunoassays rely on antigen–antibody interactions, which may hinder assay specificity. Steroids are very small molecules with little immunogenic capacity that slightly differ in their carbon backbone. Thus, molecules structurally similar to the analytes of interest can bind to the antibodies, producing false positives. Immunoassay manufacturers provide tables with percent of cross-reactivity. However, this may not be a major concern when cross-reacting molecules are not present in biological samples or only in very low concentrations.

In LC-MS/MS, multiple criteria are used as quality control for unambiguous identification of the analyte. First, the LC separates the steroids in the sample at specific retention times. When samples are injected, the analytes interact with the analytical column and then a mobile phase is applied to make the analytes elute based on the affinity with these two. Multiple parameters (e.g., column type, solvent types, flow rate, solvent gradient) can be adjusted to improve analyte separation. Second, the mass spectrometer precisely selects the analytes of interest. After chromatographic separation, the analytes are ionized, and only specific ions are selected based on their mass-to-charge ratio. Lastly, the ionized analytes are fragmented, and specific product ions are selected in the last mass analyzer. The transitions of two product ions (quantifier ion and qualifier ion) per analyte are monitored, and the analysis of the ion ratio between the quantifier and qualifier ions is calculated and used to verify the identity of the analyte. When using LC-MS/MS, numerous studies report lower steroid concentrations than studies using immunoassays, which might reflect the much greater specificity of LC-MS/MS over immunoassays.

2.10. Number of Analytes

One of the major advantages of LC-MS is that it allows for the measurement of multiple analytes in a single sample. The extracted sample is injected into the LC that physically separates the steroids of a sample with multiple components. The degree of separation is determined by the chromatographic conditions. Then, analytes are introduced into the MS

to determine the identity of the individual components. On the other hand, immunoassays rely on the use of antibodies to the target steroid of interest, allowing the measurement of only 1 steroid per assay. Because the levels of multiple steroids can change concomitantly, it is crucial to study the complete set of metabolites in a biological sample. In this way, LC-MS provides a more comprehensive assessment of steroid levels, relative to a few steroids that can be assessed with immunoassays.

3. Discussion and Conclusions

Quantifying steroid concentrations within the placenta, maternal, and fetal serum, in humans and other species (rodents are primarily discussed here) can help us understand how steroid levels and reproductive complications are related and regulated, potentially providing insight into the underlying pathophysiology of these complications. While it has long been understood that correct levels of steroids are vital in maintaining a successful pregnancy, most studies focus on the metabolism and transport of steroids rather than their absolute concentrations. The placenta presents a valuable tissue for studying steroid levels in pregnancy. It is regarded as medical waste, making it easier to collect than other biological samples. However, speed of collection and processing are important considerations for analyses of steroids as well as steroidogenic enzymes. Delays in collection can occur if physicians or pathologists need to examine the tissue following delivery, but these delays can affect experimental outcomes in the future. While processing and storage conditions will depend on the type of work to be performed on the tissue and will not be the same for all research groups, clarity surrounding collection, processing, and storage is needed. Biobanks provide valuable sample repositories for research and can also serve to standardize a protocol for tissue collection, processing, and storage. In all studies, details surrounding tissue collection should be included for transparency and interpretation of the results.

As mentioned above, tissue collection and processing are critical for good placental samples; in fact, this is true of all experimental tissues—human or otherwise. The placenta contains multiple nucleases and proteases, necessitating collection as quickly as possible [23]. We have had the most success with placentas collected from humans and rodents immediately post-birth (within minutes), but can confirm that processing times as long as 8 h have been sufficient for chemical, steroid and protein detection, quantification, and activities, although within 2 h is optimal for studying DNA, mRNA, and signaling molecules/pathways [18,19,34–36,38,39].

Additionally, analytical considerations when dealing with placentas and other reproductive tissues are more complex than simple matrices such as plasma, serum or urine. Invariably the extraction and cleanup procedures employed require LLE or SPE to completely purify the analytes of interest, particularly steroids, which holds true across humans and several other species [10,25,29,38,40]. Additionally, for LC-MS detection of steroids, detection of the more fat-soluble species, in particular estrogens, may be achieved without derivatization [10]; however, for ultrasensitive detection, derivatization may be desirable [29,41]. Finally, there are multiple examples from our own studies (see Table 1) as well as the literature showing that LC-MS is superior in being able to simultaneously detect multiple analytes simultaneously. Although most ELISAs have single analyte detection, some multiplex ELISA platforms do exist, and although they are very expensive, we have direct evidence that they can be of use in human placentas and reproductive tissues but that matrix effects occur and analyte (including steroid) detection can lack sensitivity [28,42,43].

Table 1. Results of multiple testing modalities. Human and rodent reproductive tissues tested for steroids and metabolites. LLOQ: lower limit of quantitation, LOS: limit of sensitivity.

Tissue	Steroids/Metabolites	Method	Linear Range	LLOQ	Refs.
Human placenta	Pregnenolone, progesterone, DHEA, androstenedione, testosterone, estrone, estradiol, estriol, cortisol	UHPLC-MS/MS	0.02–100 ng/mL for steroids	0.02 ng/mL	[26]
Human placenta	Progesterone, estrone, estradiol	ELISA	0.3–60 ng/mL, 10–2000 pg/mL, 20–3200 pg/mL, respectively	0.3 ng/mL, 10 ng/mL, 20 ng/mL, respectively	[30]
Human maternal plasma/fetal serum	Pregnenolone, progesterone, DHEA, androstenedione, testosterone, estrone, estradiol, estriol, cortisol, DHEAS, estrone-S	UHPLC-MS/MS	0.02–100 ng/mL for steroids, 5–1500 ng/mL for DHEAS, 2–600 ng/mL for E1S	0.02–5 ng/mL, depending on the analyte	[26]
Mouse placenta	Progesterone, estrone, estradiol, estriol	ELISA	0.3–60 ng/mL, 10–2000 pg/mL, 20–3200 pg/mL, 0.9–2000 pg/mL, respectively	0.3 ng/mL, 10 ng/mL, 20 ng/mL, 0.9 ng/mL, respectively	[30–33]
Mouse maternal/fetal serum	Progesterone, estrone, estradiol, DHEAS, androstane-3α-17β-diol glucuronide	ELISA	0.3–60 ng/mL, 10–2000 pg/mL, 20–3200 pg/mL, 0.1–10 µg/mL, 0.25–50 ng/mL, respectively	0.3 ng/mL, 10 ng/mL, 20 ng/mL, 0.1 µg/mL, 0.25 ng/mL, respectively	[31,32]
Rat placenta and decidua	Allopregnanolone, androstenedione, corticosterone, 11-dehydrocorticosterone estrone, progesterone, testosterone	UHPLC-MS/MS	0.4–140 ng/g, 0.02–2.5 ng/g, 0.01–60 ng/g, 0.01–70 ng/g, 0.01–0.3 ng/g, 0.01–7 ng/g, 0.01–0.35 ng/g, respectively	0.4 ng/g, 0.02 ng/g, 0.01 ng/g, 0.01 ng/g, 0.01 ng/g, 0.01 ng/g, 0.01 ng/g, respectively	[27]

Although several methodologies for quantifying steroid levels exist, MS is regarded as the gold standard approach. For the detection of fat-soluble molecules, including steroids, GC-MS may be superior, particularly if these molecules are not derivatized, but the use of LC-MS is also highly effective. Despite the fact that ourselves and others (referenced above) have successfully used ELISA technologies, where possible, the use of LC-MS or GC-MS is preferable if time and cost permit, and instruments as well as technical expertise are available. Here, we have outlined some of the technical and methodological considerations that need to be made when quantifying steroids in placental tissue, and sera from maternal or fetal donors. We have demonstrated good results in terms of sensitivity, selectivity, linearity, and matrix effects for UHPLC-MS/MS methods in both human and rodent placental tissues, but highlight that careful validation is required for different species and matrices to ensure reliable results. For human placenta and sera, high steroid levels, in addition to large tissue samples, mean sensitivity is rarely an issue, but this is a critical consideration in studies of rodent placenta and sera, depending on the steroid and sample size. Improvements in steroid extraction and derivatization techniques in the last several years mean very small amounts of steroids can be quantified, and with appropriate modifications, LC-MS methods can be successfully applied for both human

and rodent tissues, as well as other species. Here, we have provided technical advice on the best practices for tissue handling and analytical considerations for studying steroids in human and rodent reproductive tissues, focused on placenta. This advice stems from over 20 years of work in our laboratories with the aim of providing resources for other investigators to reduce time and cost, while increasing the robustness and validity of the experimental outcomes.

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