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Review

The importance of estradiol measurement in patients undergoing in vitro fertilization

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ABSTRACT

Successful outcomes of in vitro fertilization (IVF) are dependent in part on successful oocyte maturation and retrieval during a controlled ovarian stimulation process, which is guided by serial ultrasound and estradiol measurements. Yet, laboratory analysis of estradiol poses challenges due to the need for accuracy and specificity across concentrations that span multiple orders of magnitude. The Endocrine Society released a 2013 position statement that called for improvements in methods to analyze estradiol, and while some progress has been made in standardization and assay specificity, further work is needed to meet the needs of patients in both the IVF setting and in other clinical contexts.

This review highlights the capabilities and challenges of current laboratory methods for the analysis of estradiol in the IVF setting, including automated immunoassays and liquid chromatography-tandem mass spectrometry, and discusses current efforts to improve the analytical sensitivity and standardization of estradiol assays. Clinical laboratorians should be aware of the limitations of current estradiol assays and select appropriate methods for the measurement of estradiol in their patient population.

1. Background

In vitro fertilization (IVF) represents a successful reconstruction of early processes involved in human reproduction. IVF provides a means to circumvent an array of reproductive pathology, and now accounts for 1–2% of all live births in the United States [2]. Progressive refinements in clinical and laboratory IVF techniques have resulted in improvements in reproductive outcomes. Particularly, improvements in hormonal assays have played an important role in enhancing the clinical assessment of patients and guiding clinical decisions throughout treatment [3].

Controlled ovarian hyperstimulation (COS) is often the initial step in IVF and mimics the endogenous hormonal signaling cascade that induces follicular recruitment and maturation, as summarized in Fig. 1.

Spontaneous menstrual cycles begin with a quiescent state where both gonadotropin hormone concentrations and ovarian production of estradiol and progesterone are low. The return of ovarian hormone concentrations to baseline releases the hypothalamus and pituitary from feedback inhibition, resulting in a gradual rise in gonadotropins and thus follicular recruitment. With continued follicular growth, there is a corresponding rise in estradiol concentrations, which serves as a surrogate marker for oocyte maturity. Rising concentrations of estradiol act on the hypothalamus and pituitary via negative feedback to decrease gonadotropin concentrations. The drop in gonadotropin concentrations precludes further follicular recruitment, but sustains growth of larger follicles given their greater density of gonadotropin receptors. This promotes selection of one dominant follicle in vivo [2]. Once estradiol concentrations rise above a critical threshold, they act via positive feedback to abruptly increase gonadotropin release from the pituitary, thereby inducing ovulation [2].

During COS, daily administration of exogenous gonadotropins via injectable medications overrides negative feedback from rising endogenous concentrations of estradiol, which induces multi-follicular recruitment and maturation [2]. Gonadotropin releasing hormone antagonists are used during the stimulation cycle once follicular recruitment has been established to prevent endogenous surge of gonadotropins, which prevents premature ovulation. The COS process normally varies from 8 to 12 days, and requires serial monitoring with serum estradiol concentrations and pelvic ultrasound every 1-2 days [2,4]. This laboratory evaluation and imaging provides critical information on the response to treatment and presumed maturity of oocytes. When the largest cohort of follicles are presumed mature, patients administer the final injection with either human chorionic gonadotropin hormone or leuprolide acetate, mimicking the abrupt

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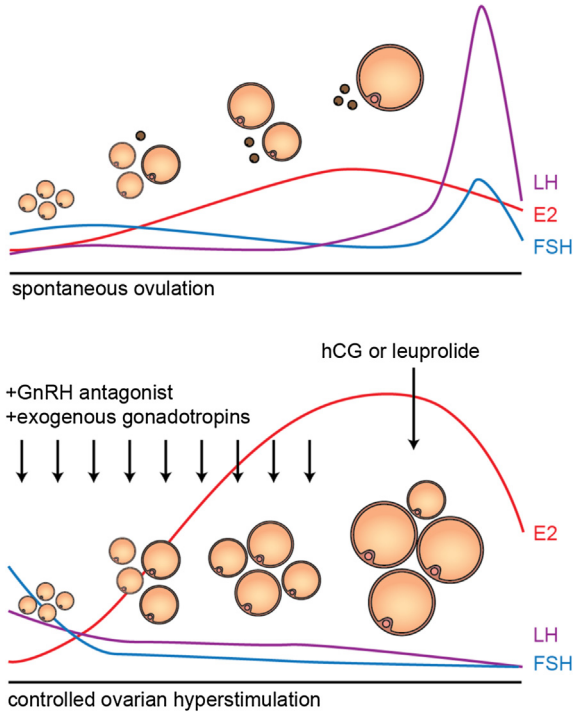

Fig. 1. Schematic of follicular maturation in spontaneous cycles (single dominant follicle recruitment) and during controlled ovarian hyperstimulation (multi-follicular recruitment). Patterns of estradiol (E2), LH, and FSH are highlighted with respect to the growth of follicles.
Adapted from [1]

surge in luteinizing hormone or stimulating endogenous luteinizing hormone release respectively. This results in the final steps necessary to complete oocyte maturation and ovulation. Oocytes are then retrieved prior to ovulation and combined with sperm in the lab to facilitate fertilization. The embryos are monitored during early growth and development with eventual placement into the uterine cavity.

Clinicians rely on the accuracy of estradiol measurements to correlate with radiographic findings during the assessment of follicular growth and oocyte maturation throughout COS. This directly influences decisions regarding medication dosage, length of stimulation, and presumed timing of trigger for retrieval of oocytes. In addition, estradiol concentrations are used as a predictive marker for development of ovarian hyperstimulation syndrome (OHSS). OHSS is one of the major complications of COS, and results from an exaggerated response to gonadotropins. This causes the upregulation of growth factors that drive increased vascular permeability. Patients may develop ascites, and may present with laboratory abnormalities suggestive of hemoconcentration and/or end-organ hypoperfusion. Elevated peak estradiol levels during COS are a risk factor for the development of OHSS, with other risk factors including age, serum anti-Müllerian hormone concentration, and increased number of follicles observed by ultrasound [5].

Thus, estradiol concentrations are critical in assessing patient risk for OHSS and monitoring response to treatment. Clinical decisions made during COS impact oocyte yield during retrieval and may have downstream implications on embryogenesis.

2. Laboratory measurement of estradiol

Measurement of estradiol poses several challenges due to the biochemistry and physiology of this hormone. Estradiol is one of a large family of structurally-related estrogens and must be detected specifically, and hormone concentrations must be measurable with high accuracy and precision across a wide range of concentrations (approximately 1–3000 pg/mL). This wide range enables utility of estradiol assays across multiple clinical applications, ranging from COS on the high end of the spectrum and therapeutic monitoring of aromatase inhibitor treatment in men and postmenopausal women on the low end.

2.1. Biochemistry of estradiol

17β-Estradiol is an 18-carbon steroid hormone produced from the precursor androgens androstenedione and testosterone, largely in ovarian theca interna cells. The enzyme aromatase carries out a series

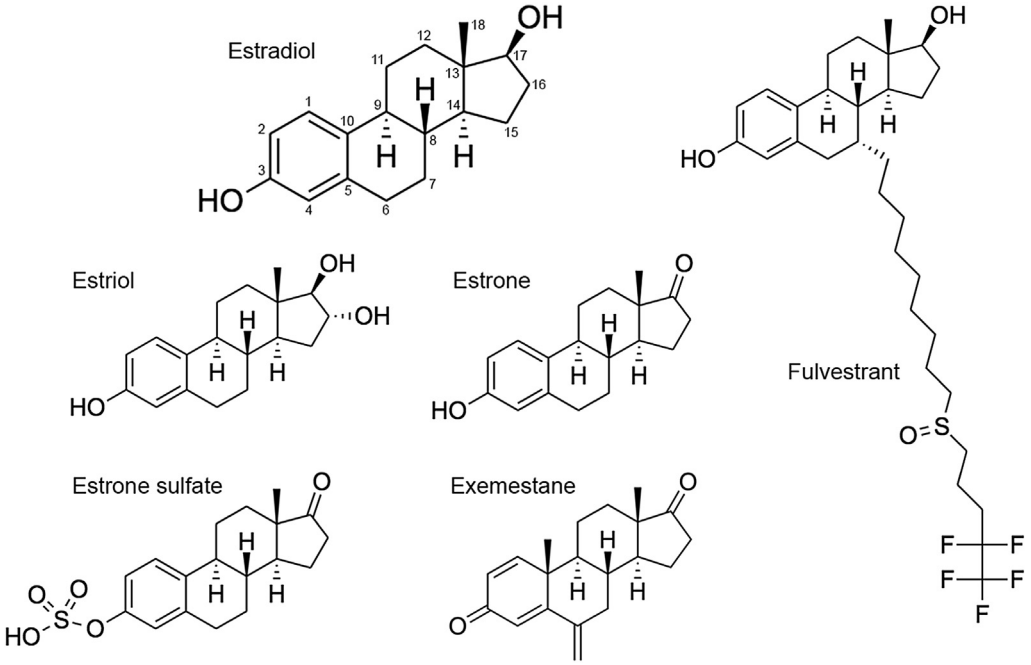

Fig. 2. Structures of estradiol and closely-related compounds. Some medications (exemestane, fulvestrant) that have been reported to cause interference in estradiol assays are also shown.

of reactions that yield estrone and estradiol, with 17β-hydroxysteroid dehydrogenase interconverting between the two [6]. Estradiol is the major estrogen product of the ovaries and also exerts the most potent estrogenic activity, although further metabolism (through oxidation, sulfation, or glucuronidation) yields a large number of other estrogens, estimated to be at least 20 unique species [6]. While estradiol is typically the most abundant circulating estrogen, there is potential for other estrogens to drive positive interference in assays based on overlap in antigen specificity or molecular weight. The structures of estradiol and example related estrogens are highlighted in Fig. 2.

Estradiol, like other steroid hormones, is extensively protein-bound in circulation. Only 2-3% of estradiol circulates freely, while 40–60% of the total pool in women circulates bound to sex hormone binding globulin (SHBG). The remainder of circulating estradiol is bound to albumin through a nonspecific interaction [6]. In contrast to measurement of testosterone, where free or bioavailable fractions are reported clinically, estradiol is only measured as a total pool. Thus, an important consideration for measurement of total circulating estradiol is complete and reproducible dissociation of estradiol from albumin and sex hormone binding globulins (SHBG) during sample preparation.

2.2. Contemporary methods for measurement of estradiol

Historically, measurement of estradiol was performed by radioimmunoassay, and later by gas chromatography-mass spectrometry (GC-MS). Today, the major methods used for clinical measurements of estradiol are chemiluminescent immunoassays, and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). For women undergoing assisted reproduction, in order to time human chorionic gonadotropin (hCG) administration and to monitor treatment to minimize the risk of OHSS, a concentration range spanning from 10 to 2000 pg/mL is needed [7].

2.2.1. Immunoassays

Immunoassays have long been the standard method used for measurement of estradiol, and automated immunoassays are presently the dominant method used for routine laboratory measurements of estradiol. In a 2018 CAP survey of estradiol methods, 99% of respondents were using automated immunoassay methods [8]. The majority of these methods are competitive binding immunoassays using chemiluminescent detection, with a displacing agent such as an androgen or estrogen analogue added to the reagent cocktail to permit direct measurement of estradiol without an initial extraction step. Some characteristics of commonly used automated immunoassays are summarized in Table 1.

Immunoassays are attractive for a number of reasons in the setting of estradiol measurement in COS. Modern estradiol immunoassays require little hands-on work from laboratory technologists and are relatively rapid and inexpensive to perform, enabling laboratories to meet turnaround time demands for serial monitoring of hormone concentrations [17]. Further, most automated estradiol immunoassays have good precision and are reasonably accurate in the concentration ranges expected in COS (> 200 pg/mL) [9]. However, for high estradiol concentrations, serial dilution is often required. This may cause delays in turnaround time, represents an opportunity for the introduction of error from manual pipetting steps, and can also introduce error due to matrix effects from sample diluent.

Assay standardization has long been a challenge for hormone measurements by immunoassay. Differences in antibody specificity and affinity represent an obvious source of variation between assays, but other sources of variation might include matrix differences between calibrator and control material between assays or variation in the extent and reproducibility of estradiol release from binding proteins. The non-commutability of estradiol immunoassays with one another has long been recognized, but recent publications highlight the ongoing nature of this problem. Zhang and colleagues reported a comparison between the Architect i2000SR (Abbott, Abbott Park, IL), Cobas e601 (Roche, Basel, Switzerland), and UniCel DxI 800 (Beckman Coulter, Brea, CA) methods that showed good correlation but poor numerical agreement between the three methods [18]. Peavey and colleagues performed a similar study to compare the Architect i1000 (Abbott, Abbott Park, IL) and ADVIA Centaur CP (Siemens, Malvern, PA) immunoassays with an ABSciex 5500 (Sciex, Framingham, MA) LC-MS/MS method, and found positive bias of 20% between the ADVIA and both Architect and LC-MS/MS assays, while the Architect did not exhibit significant bias from LC-MS/MS [19]. Results from a 2018 CAP survey of estradiol methods also highlights the wide variation among assays – the coefficient of variation for the set of all methods was 23.3% for a sample with a mean of 137.1 pg/mL estradiol (range of 97.1–318.4 pg/mL) [8].

Given that more than 100 conjugated and unconjugated estradiol metabolites and structurally similar exogenous hormones (as in Fig. 2) may be present in blood to interfere with the measurement [20], a specific assay of estradiol is desired. Assay specificity for estradiol has improved with recent-generation immunoassays. Sluss and colleagues demonstrated absence of significant interference from estrone (> 90% recovery of estradiol with 750 and 4000 pg/mL estrone) and estriol (> 90% recovery at 150 and 4000 pg/mL) on the Architect i2000 assay [10]. Krasowski and colleagues demonstrated no significant cross-reactivity of the estradiol assay on the Roche Elecsys and Modular E170 analyzers (Roche, Basel, Switzerland) with estrone (0.54% cross-reactivity), ethinyl estradiol (0.23% cross-reactivity) or estriol (0.09% cross-reactivity) [21]. Yet, other reports have indicated that endogenous and exogenous interferences can still cause inappropriate immunoassay measurements. Hosokawa and colleagues highlighted a case of discrepant estradiol measurements by Roche Elecsys E2III (Roche, Basel, Switzerland) assay relative to LC-MS/MS in a patient with an ovarian teratoma [22]. Biotin interference causing apparent hyperestrogenism has been reported for the Vitros 5600 (Ortho Clinical Diagnostics, Raritan, NJ) estradiol assay in a case published by Batista and colleagues [23]. Other cases have indicated falsely elevated estradiol from fulvestrant therapy with the Architect assay [24] and exemestane therapy with the Architect i1000 and Roche Cobas E411 (Roche, Basel, Switzerland) [25].

Currently, immunoassays have insufficient analytical sensitivity and precision at low measurement ranges to be appropriate for measurements in children, men, and postmenopausal women. Even for assays that exhibit good agreement with LC-MS/MS methods at higher

Table 1
Summary characteristics of common estradiol automated immunoassays. Assay manufacturers are Abbott (Abbott Park, IL), Beckman Coulter (Brea, CA), Roche (Basel, Switzerland), Siemens (Malvern, PA), and Ortho Clinical Diagnostics (Raritan, NJ).

Method	LOQ (pg/mL)	Linear range (pg/mL)	Total imprecision	Source
Abbott Architect i	< 18		1.2–9.9%	[9]
	< 15	15–4000	2.3–7.1%	[10]
	14	25–1000	1.8–7.4%	[11]
Beckman Access/2	37		3.3–14.7%	[9]
	15	15–5200	3–9%	[12]
Beckman Unicel DxI	15	15–5200	3–9%	[12]
Roche Cobas e411/ Elecsys	< 18		3.6–8.9%	[9]
	25	25–3000	2.5–11.9%	[13]
Roche Cobas e600/E170	25	25–3000	1.9–10.6%	[13]
Siemens ADVIA Centaur CP eE2	18	18–3000	2.9–14.5%	[14]
Siemens ADVIA Centaur/ XP/XPT	61		6.8–42.6%	[9]
Siemens Dimension Vista	11	11–1500	1.8–15.6%	[15]
Siemens Immulite 2000/ Xpi	80		4.4–21.0%	[9]
Ortho Vitros 3600, 5600, Eci, ECiQ	< 18		2.5–8.9%	[9]
	6	6–3183	3.2–11.5%	[16]

measurement ranges, agreement at lower concentrations (< 100 pg/mL) is poor [8,19,26]. Accuracy of immunoassays with respect to mass spectrometry also appears to suffer at very high concentrations of estradiol that are of relevance in COS (> 500 pg/mL) [9], and better accuracy and precision at these high concentrations will be important for improving outcomes in IVF. Measurement of very high concentrations of serum estradiol is an important component of the prediction of OHSS [27]. Yet, published cutoff values for prediction of OHSS vary widely [28–30], possibly due to the variation of immunoassays by manufacturer and at high concentrations. Emerging research suggests that detecting changes in estradiol at high concentrations could also be a useful predictor of IVF outcomes [27,31,32], but accurate classification of patients for further research will also rely on the development of more accurate assays and universal cutoff values.

2.2.2. Mass spectrometry

Mass spectrometry is already widely used for measurement of some steroids, as in the investigation of inborn errors of steroidogenesis. Other steroid measurement programs are slowly beginning to transition to mass spectrometry-based methods, given the higher sensitivity and specificity of these instruments for small analytes that are difficult to target with antibodies [17]. Mass spectrometry systems are typically coupled to chromatographic separation, such as gas chromatography or liquid chromatography. Of these, liquid chromatography (LC-MS/MS) is the most commonly used in contemporary systems, with the liquid chromatography steps performed by high performance liquid chromatography (HPLC) or ultra-high performance liquid chromatography (UHPLC). The current Centers for Disease Control and Prevention (CDC) reference method for estradiol is isotope dilution high performance liquid chromatography tandem mass spectrometry [33].

The use of mass spectrometry-based methods for estradiol is becoming more widely adopted, with over 50 publications on the subject indexed by PubMed.gov every year since 2011, and a 2018 CAP survey for estradiol indicated that 22 (out of 1484) respondents were using mass spectrometry methods [8]. The proliferation of well-developed mass spectrometry methods for other clinical purposes, such as therapeutic drug monitoring and diagnosis of inborn errors of metabolism, as well as the introduction of mass spectrometry-based standardization programs for testosterone and Vitamin D [34], has laid groundwork for more widespread adoption of mass spectrometry for measurement of estradiol.

As with immunoassays, one of the challenges facing mass spectrometry measurement of estradiol is assay standardization. As laboratory-developed tests, mass spectrometry methods often exhibit substantial variation in sample preparation, sample matrix and ion suppression effects, and calibration between laboratories. The 2018 CAP survey for estradiol highlights this issue – among all methods used by respondents, mass spectrometry (reported as a single method) exhibited the highest coefficient of variation between laboratories at both low and high concentrations (16.5% at a mean of 126.3 pg/mL and 14.1% at a mean of 2277.3 pg/mL, relative to a mean CV of 7.5% and 5.8% for all other methods) [8]. Mass spectrometry methods are amenable to standardization due to the laboratory-developed nature of the assays – in contrast to immunoassays, which would require reclassification as laboratory-developed tests if recalibrated to a new universal reference material. However, there is a paucity of studies to compare assay performance characteristics of mass spectrometry methods. Some characteristics of tandem mass spectrometry methods published in the past five years are summarized in Supplemental Table 1.

Mass spectrometry methods have some technical challenges not shared by immunoassays. Sample preparation is often laborious, requiring extraction or derivatization steps that must usually be performed manually and which vary from laboratory to laboratory. Sample derivatization methods are particularly variable. Most recent reports indicate derivatization with dansyl chloride, while other derivatization methods include 1-(5-fluoro-2, 4-dinitrophenyl)-4-methylpiperazine (PPZ) [35], 2-fluoro-1-methylpyridinium-p-toluenesulfonate (FMP-TS) [36], 3-[(N-succinimide-1-yl)oxycarbonyl]-1-methylpyridinium iodide [37], 1,2-dimethylimidazole-5-sulfonyl chloride [38], and N-methyl pyridinium-3-sulfonyl derivatization [39]. Almost as many authors reported measurement of estradiol without derivatization, and there are conflicting reports regarding whether derivatization is useful to improve detection of estradiol. Ke and colleagues reported that derivatization with dansyl chloride improved the sensitivity of LC-ESI-MS for estradiol [40], while Boggs and colleagues reported increased response of estradiol by LC-MS/MS as well as improved chromatographic separation of derivatized estrogens [41]. Meanwhile, Botelho and colleagues reported that the specificity of their method for estradiol was improved without derivatization [42].

Automated instrumentation may be a solution to reducing the time spent on these manual steps, but is expensive to implement. While mass spectrometry systems typically have higher throughput than radioimmunoassays, they suffer in comparison to automated immunoassays. HPLC and UHPLC systems reduce the run time for chromatographic separation upstream of mass spectrometry detection, and are most commonly used in contemporary systems for this reason. System maintenance, calibration, and proficiency testing are also more complicated than for immunoassays due to the manual and laboratory-developed nature of the method.

Perhaps the biggest barrier to wider implementation of mass spectrometry for measurement of estradiol is the associated cost. Chromatography and mass spectrometry systems that are sufficiently sensitive to detect estradiol across all physiologically relevant ranges are expensive, so the initial cost is high for laboratories without an existing instrument. Additionally, the technical expertise required to maintain a mass spectrometry program is substantial, so more skilled personnel are needed. In addition, the time to result is increased for tandem mass spectrometry methods compared to immunoassays.

3. Paths forward for measurement of estradiol

In 2013, the Endocrine Society published a position statement on the measurement of estradiol, calling for improved sensitivity, specificity, accuracy, and precision for estradiol assays [43]. While there has been some progress made to address the issues raised by this statement since its publication (as in improvements in assay specificity discussed in Section 2.2.1.), further challenges remain.

The Endocrine Society position statement recommends the development of new methods with sufficient sensitivity and precision to monitor estradiol concentrations lower than 2 pg/mL, as in the case of aromatase inhibitor therapy [43]. Radioimmunoassays have a superior limit of quantitation (LOQ, the lowest concentration that can be measured with a coefficient of variation of 20%) at ~1 pg/mL, but are no longer widely used. Typical LC-MS/MS methods and 2nd generation immunoassays offer LOQ of ~10 pg/mL, while 1st generation immunoassays have LOQ of ~30 pg/mL [20,44]. Multiple novel immunoassay methods have been developed that report LOQ at or near the 2 pg/mL threshold [45–48], and several LC-MS/MS methods have reported LOQ of less than 1 pg/mL [38,39,49], but further work will be necessary to make these or other sufficiently sensitive methods widely available for clinical use. Additionally, per Endocrine Society recommendations, methods must also be accurate at the highest estradiol concentrations seen in COS (approximately 3000 pg/mL) [43]. However, only a few of the LC-MS/MS methods reported in recent literature with estradiol LOQ at or below 2 pg/mL also have reported linearity above 1000 pg/mL (Supplemental Table 1), so further work must keep in mind the need for accurate measurement of estradiol in IVF populations.

The position statement also calls for improved accuracy and commutability of estradiol results among laboratories, which will ultimately permit development of universal age- and population-specific reference ranges. A major hindrance to this goal is the absence of a

Table 2
Features of laboratory methods for measurement of estradiol.

Estradiol assay method	Benefits	Challenges
Radioimmunoassay	• Low limit of detection and limit of quantification • Historical gold standard	• Manual steps, some methods require pre-extraction • Radioisotope usage • Low throughput • Antibody cross-reactivity
Automated immunoassay	• High throughput • Inexpensive • Suitable accuracy for most estradiol concentrations seen in women • Widely available	• Poor analytical sensitivity and accuracy at extreme low concentrations • Poor accuracy at extreme high concentrations • Non-commutability between manufacturers • Assay interference from medications
LC-MS/MS	• Modern gold standard • Improved specificity • Greater throughput than radioimmunoassays • Flexible platform, amenable to assay development and standardization • Low detection limits	• Wide method variability • High up-front cost • Technically demanding – laboratory-developed test • Many manual steps • Lower throughput than automated assays

universal estradiol reference material – present studies refer to multiple different estradiol reference materials, including the European Commission Joint Research Center BCR 576, 577, and 578 [50,51]. A single reference material to which all estradiol assays are traceable will greatly improve the ability to standardize methods.

To aid in estradiol assay standardization, the Centers for Disease Control and Prevention (CDC) Hormone Standardization (HoSt) Program was initiated for estradiol in 2014 [52]. This program focuses on producing single donor human matrix-matched materials for commutability, developing a reference system, calibrating different assays to a single reference material, and certifying individual test performance based on accuracy and precision [53]. The HoSt program is targeted towards assay manufacturers and laboratory-developed tests, and grants certification to methods that achieve bias of 12.5% or less at estradiol concentrations of > 20 pg/mL, and bias of 2.5 pg/mL or less at ≤ 20 pg/mL for at least 80% of samples [33]. At present, 12 LC-MS/MS methods hold current or past HoSt certification for measurement of estradiol, but only one immunoassay method (Fujirebio Lumipulse, Malvern, PA) is currently certified [54]. Increasing participation in the HoSt program could help drive standardization of estradiol assays, as has previously been performed successfully for testosterone and for vitamin D. However, this program is costly, and it only certifies assays in the range of 1.5–210 pg/mL estradiol, which is a narrower measurement range than recommended by the Endocrine Society.

4. Conclusions

Laboratory measurement of estradiol is a key component of the management of IVF patients, as it is critical for guiding the timing and dose of gonadotropin administration in COS. For this application, automated immunoassays remain the dominant assay method, and in the years since the Endocrine Society released its position statement on the measurement of estradiol, multiple studies have demonstrated improvements in the specificity of these assays for estradiol and their agreement to LC-MS/MS reference methods. However, further challenges remain, as automated immunoassays still exhibit poor agreement with one another and may not be accurate for measurements at the extreme ends of physiological concentration ranges for estradiol. Use of LC-MS/MS methods offers opportunities for improved analytical sensitivity and specificity for estradiol, and is especially amenable to standardization, but the financial and technical commitment remains a significant barrier for widespread adoption. Further, the absence of a universal standard reference material for estradiol hampers efforts towards assay standardization and reference range generation. Some of these points are summarized in Table 2.

Thus, until issues of standardization, analytical sensitivity, and measurement accuracy are addressed for estradiol measurement by automated immunoassays, it is prudent to use a method that has been standardized to the gold standard method, such as LC-MS/MS and be cognizant of two points: (1) different immunoassays may yield different results and thus a patient needs to be followed using a single method and (2) manufacturers of immunoassays must attempt to standardize their methods to values obtained using the CDC HoSt program. Optimal outcomes for patients undergoing IVF relies on the accuracy of estradiol measurements, along with that of other hormones, and it is the responsibility of laboratorians to ensure this accuracy of measurement.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.09.021.

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