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Review

Estradiol assays – The path ahead

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ABSTRACT

Estradiol quantitation is useful in the clinical assessment of diseases like hypogonadism, hirsutism, polycystic ovary syndrome (PCOS), amenorrhea, ovarian tumors and for monitoring response in women receiving aromatase inhibitor therapy. Physiologically relevant serum estradiol concentration in women can span across four orders of magnitude. For example, in women undergoing ovulation induction serum estradiol concentration can range between 250–2000 pg/mL whereas aromatase inhibitor therapy can decrease serum estradiol concentration to <5 pg/mL. While high-through-put automated un-extracted (direct) immunoassays accommodate the growing clinical need for estradiol quantitation, are amenable to implementation by most hospital clinical laboratories, they display a significant loss of specificity and accuracy at low concentrations. Most clinical scenarios (example: estradiol monitoring in fertility treatments) place a modest demand on accuracy and precision of the assay in use but accurate quantitation of estradiol in certain clinical scenarios (pediatric and male patients and for monitoring aromatase inhibitor therapy) can be challenging using currently available immunoassays since the direct immunoassays are prone to issues with sub-optimal accuracy and specificity due to cross reactivity with estradiol conjugates and metabolites. In this review we discuss the bases for the evolution of estradiol assays from extracted (indirect) radio-immunoassays to direct immunoassays to liquid-chromatography tandem mass spectrometry (LC–MS/MS) based assays, discuss technical factors relevant for development and optimization of a LC–MS/MS assay for estradiol and present the details and performance characteristics of an ultra-sensitive LC–MS/MS estradiol assay with a limit of quantitation of 0.2 pg/mL.

Contents

1. Introduction

An increased demand for highly sensitive and specific assays for the quantitative assessment of estradiol has paralleled our increased understanding of the role estradiol plays in human physiology [1]. The enzyme, aromatase converts testosterone and androstenedione to estradiol and estrone, respectively (Fig. 1a). Quantitation of androgens and estrogens facilitates the clinical evaluation of diseases like hypogonadism, hirsutism, polycystic ovary syndrome (PCOS), amenorrhea, prostate cancer, testicular tumors and ovarian tumors.

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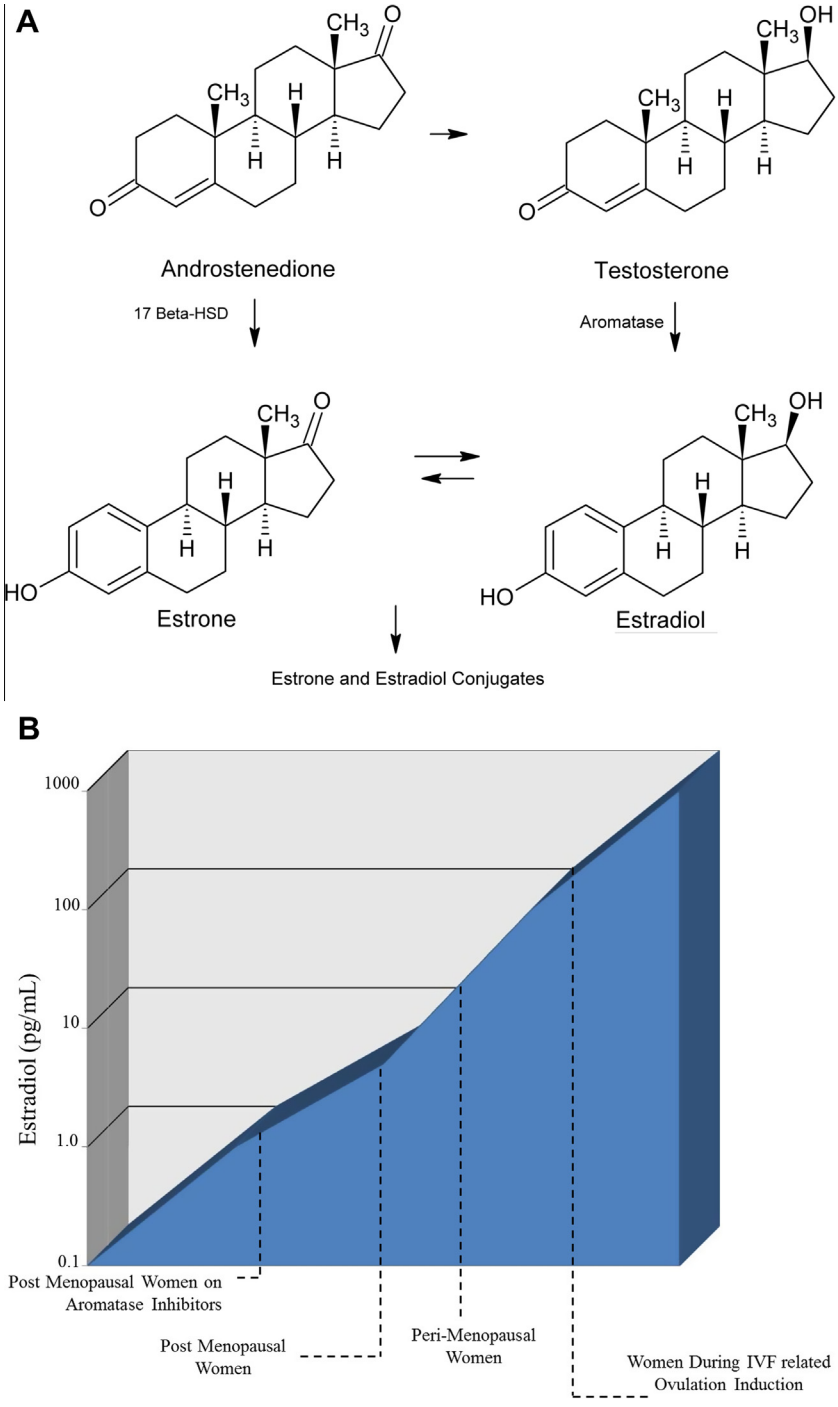

Fig. 1. (a) Biosynthesis of estradiol. (b) Range of physiologically relevant estradiol concentrations (*adapted from Ref. [2]).

Estradiol concentrations in pre-pubertal children, adolescents, pre- and post-menopausal women and in pregnant women range over several orders of magnitude (40–2000 pg/mL) [2–4]. Increased risk of breast cancer in post-menopausal women with higher circulating concentrations of estrogens and androgens has been demonstrated in prospective studies [5–7]. In women receiving aromatase inhibitor (AI) therapy for breast cancer treatment, estradiol concentrations can be 1–5 pg/mL or lower (Fig. 1b) [4,8,9]. Standardization of an assay over this wide range of concentrations is an arduous undertaking. Estradiol levels in clinical situations like the monitoring of ovulation induction as well as in preparation for in vitro fertilization, place only modest demands on assay sensitivity. However there is an increasing clinical need for high sensitivity estradiol assays for patients receiving AI therapy for breast cancer treatment, for the diagnosis of in inborn errors of sex-steroid metabolism, for pubertal disorders, for estrogen deficiency in men and for therapeutic monitoring in women receiving low dose hormone replacement therapy [10].

Assay methodologies used for estradiol quantitation include immunoassays [2,11–14], gas-chromatography mass spectrometry (GC–MS) [8,15,16] and liquid chromatography tandem mass spectrometry (LC–MS/MS) [17–20]. LC–MS/MS assays for estradiol offer better specificity and sensitivity than immunoassays [20]. Currently available LC–MS/MS estradiol assays offer the appropriate sensitivity and specificity needed for optimal management of a number of patient subsets including post-menopausal women,

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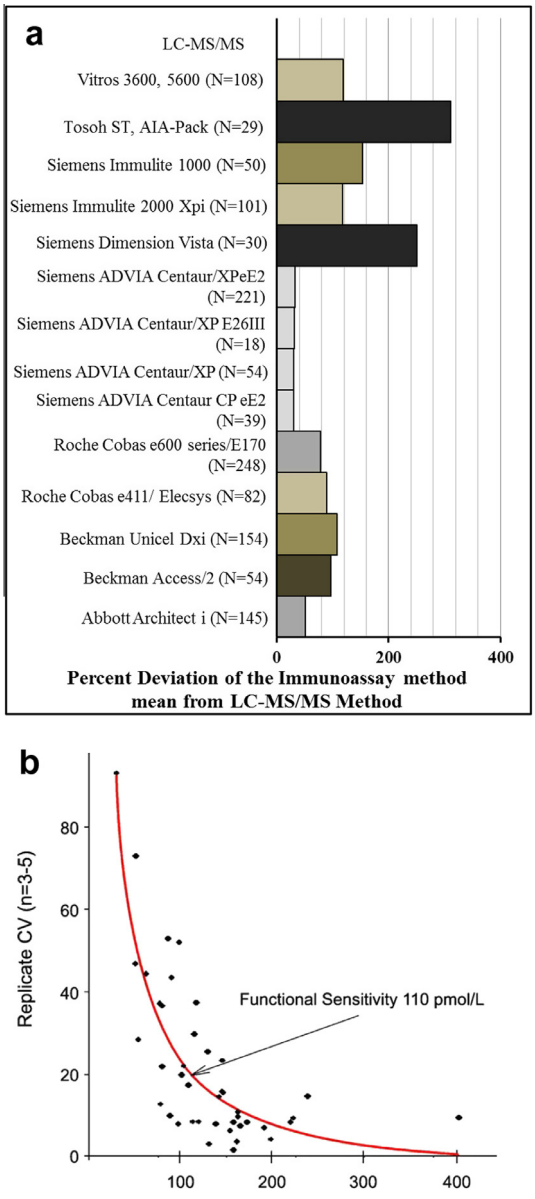

Fig. 2. (a) Percent deviation of 14 immunoassay mean concentrations from LC–MS/MS value. Number of laboratories performing each immunoassay is listed in parenthesis. Data from CAP 2013 survey (concentration using LC–MS/MS = 29 pg/mL). (b) Lack of precision at low estradiol concentration measured on AxSYM (Assembly #37000-108, Abbott Diagnostics Division, Abbott Park, IL) (*figure adapted from Dighe et al. Clin Biochem 2005, permission pending).

men and for monitoring small changes in circulating estradiol concentrations in neonates and in females receiving transdermal estradiol supplementation for the treatment of Turner Syndrome [21,22]. However many clinical laboratories still rely on automated immunoassay platforms for quantitation of estradiol as the monetary and technical investments have been limiting factors for the development and implementation of LC–MS/MS assays for routine clinical use [10,20]. Additionally high throughput capability and short turn-around times are offered by most automated immunoassay platforms that are easier to validate and implement for clinical use.

In this article we will discuss the evolution of and the challenges in the measurement of estradiol using immunoassays, the advantages offered by LC–MS/MS estradiol assays and briefly describe a high-sensitivity LC–MS/MS estradiol assay.

2. Evolution of estradiol immunoassays

Before the introduction of the first immunoassays for estradiol (1950s) bioassays were used to quantitate estradiol. There was an exponential increase in the number of radio-immunoassays (RIA) for steroids and protein hormones that became available for clinical use after Rosalyn Yalow described the first RIA for insulin in 1960 [23]. The first RIA for the quantitation of estradiol was described in 1969 [24]. In this method the serum or plasma sample had to be extracted with an organic solvent prior to quantitation by RIA. The extraction step was particularly necessary to achieve the sensitivity and to overcome analytical interferences for the quantitation of low abundance analytes like steroids. The methodology involving organic extraction prior to quantitation is referred to as an "indirect" or an "extracted" RIA. Even though RIA offers the sensitivity needed to make decisions in most clinical situations, RIA's posed several challenges to the routine clinical laboratory. RIA's required laborious extractions, the handling of radioactive materials, were subject to cross-reactivity and were prone to interferences and artifacts from the non-specific binding of radioactivity and quality control issues. Immunoassay platforms in which estradiol could be directly quantitated (also termed as "direct" immunoassays) were subsequently developed. Most direct immunoassay formats for estradiol rely on a buffer that dissociates estradiol from albumin and sex hormone binding globulin allowing for direct quantitation of estradiol in serum using highly specific antibodies. These have eliminated the need for laborious extractions, offer fast assay times, improved throughput and are amenable for use in most clinical laboratories. Most of the direct automated immunoassay platforms are optimized to measure estradiol concentrations between 40 and 2000 pg/mL, concentrations pertinent to clinical situations like in-vitro fertilization and ovulation induction. The recent Endocrine Society Position Statement has highlighted the challenges involved in the accurate and specific quantitation of estradiol over the wide range of physiologically relevant levels in diverse populations [2]. Analysis of data reported in a recent College of American Pathologists (CAP) survey showed that at an estradiol concentration of 29 pg/mL (LC–MS/MS), all 14 immunoassay platforms overestimated the estradiol

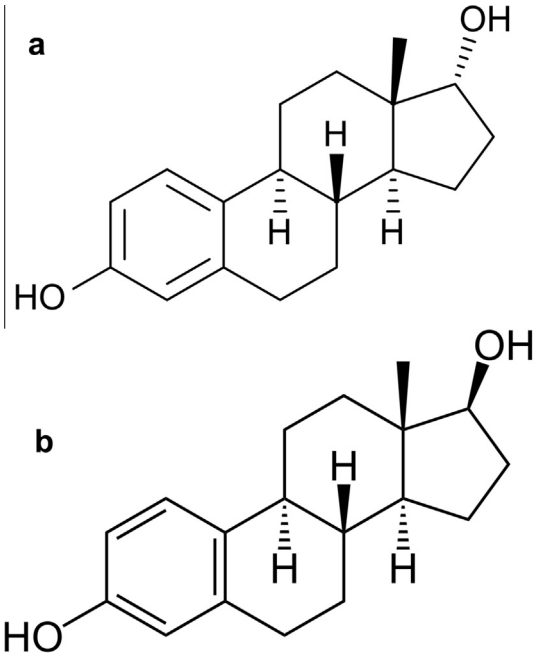

Fig. 3. Chemical structures of (a) 17α estradiol and (b) 17β estradiol.

concentration. Fig. 2a shows the percent deviation of the immunoassay mean concentration from the LC–MS/MS value. One of the immunoassays (Tosoh, ST, AIA Pack) showed a ~300% deviation from the LC–MS/MS value. Similar results have been reported by other studies [2]. Most commercially available direct immunoassays lack the performance characteristics required for the accurate and specific quantitation of estradiol in men and post-menopausal women (Fig. 2b) [14].

High variability across different direct immunoassays has been observed for estradiol measured in men and post-menopausal women [12,25]. In this patient population most automated direct immunoassays measuring estradiol concentrations fail to meet current analytical performance needs pertinent to research and patient care. A recent study comparing 11 immunoassays and 6 mass spectrometry methods with the GC–MS reference method, reported a mean bias between 2.4% and 235% in serum estradiol measurements in samples from men and pre- and post-menopausal women. A better correlation between the gold standard GC–MS/MS method and indirect RIA has been demonstrated [12].

Pharmacotherapy with third generation AIs in early breast cancer treatment lower 5-year relapse rates and delays disease progression in women with metastatic disease [26,27]. Third generation AIs suppress aromatase activity by 90–99%, resulting in a reduction of estradiol concentrations to 1–10% of pretreatment levels [8,28]. AIs significantly reduce serum estradiol concentrations preventing the growth of estrogen receptor positive metastases [28]. Optimal response during pharmacotherapy with third generation AI is dependent on the degree of estrogen suppression [28]. An estradiol concentration of 5.8 ± 4.1 pg/mL measured using an indirect RIA compared to <0.7 pg/mL using GC–MS assay was observed in women receiving AI therapy [8]. Jaque et al. also showed that commercially available estradiol immunoassay kits lack the sensitivity and specificity to measure the extremely low serum estradiol levels in postmenopausal breast cancer patients

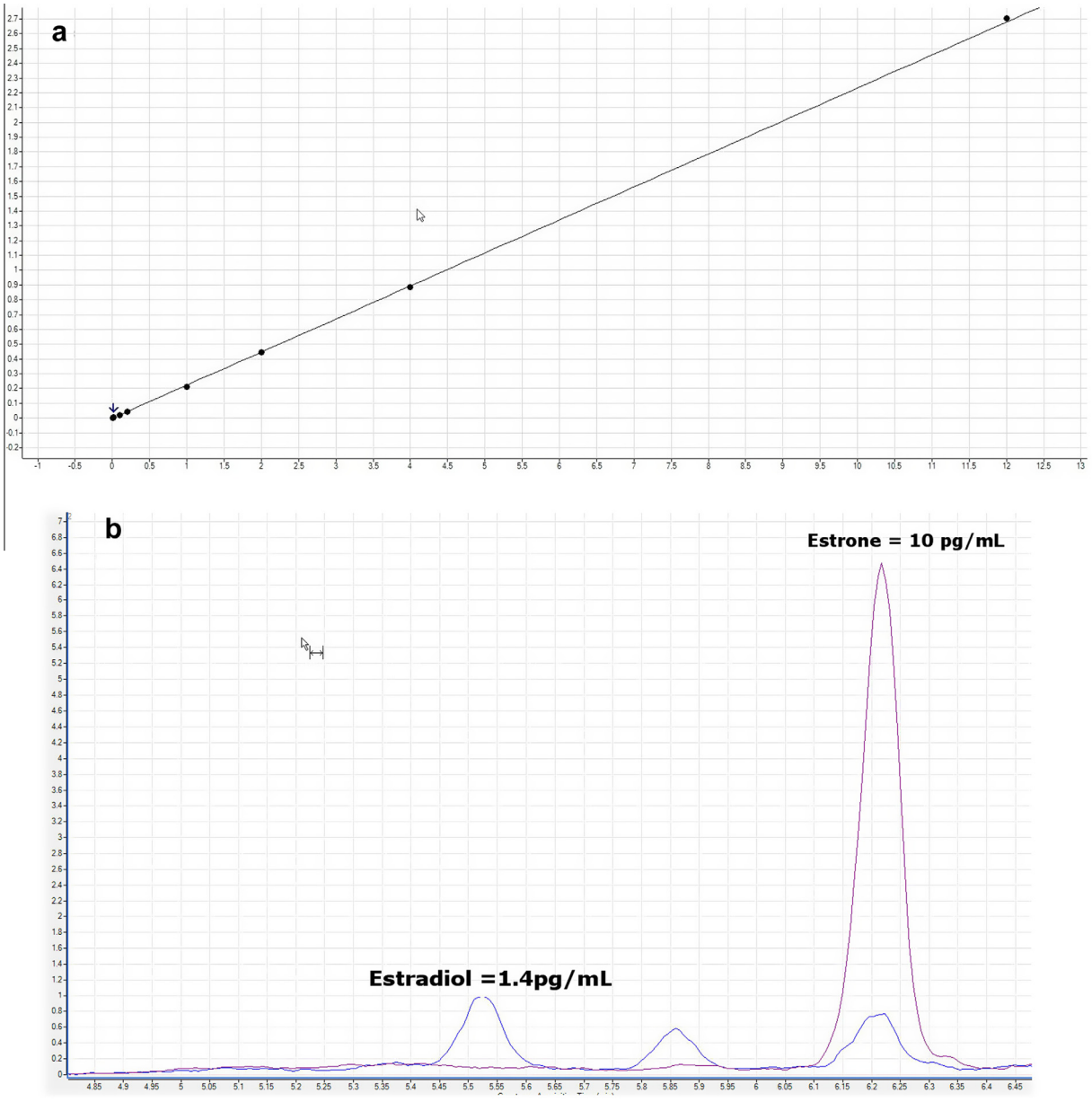

Fig. 4. (a) Calibration curve for estradiol, analytical measurable range: 0.25–600 pg/mL. (b) Chromatographic peak of a patient sample at a estradiol concentration of 1.40 pg/mL. *X* axis: counts per second × 10^6, *Y* axis: retention time in minutes.

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Table 1
Percent coefficient of variation (CV) for within run (inter-assay) imprecision ($N = 15$) with a high sensitivity LC–MS/MS estradiol assay.

Quality control levels	Estradiol mean (pg/mL)	Estradiol (% CV)
Level I	0.23	11.8
Level II	0.50	7.3
Level III	0.74	6.0
Level IV	35	1.6
Level V	151	1.5
Level VI	405	1.4

Table 2
Percent coefficient of variation (CV) for between run (intra-assay) imprecision ($N = 15$) assessed over 10 days with a high sensitivity LC–MS/MS estradiol assay.

Quality control levels	Estradiol mean (pg/mL)	Estradiol (% CV)
Level I	0.29	10.8
Level II	0.50	8.5
Level III	0.78	6.9
Level IV	32	5.1
Level V	140	4.6
Level VI	382	4.8

receiving AI treatment [13]. Indirect RIA, a more sensitive methodology compared to direct immunoassays for the measurement of low concentrations of serum estradiol has been suggested as an alternative until LC–MS/MS assays become more widely available in hospital laboratories [13].

3. The path ahead – high sensitivity LC–MS/MS estradiol assays

Assays capable of measuring estradiol levels accurately below 10 pg/mL can be termed as "high sensitivity" assays. High sensitivity assays capable of accurately measuring estradiol levels in the range of 0.2–2 pg/mL have become imperative for optimal management of patients undergoing AI therapy. High sensitivity RIA [29] and LC–MS/MS [17] methods with limits of quantitation (LOQ) of 1–2 pg/mL have been reported. However, most direct immunoassays, indirect RIAs and even some LC–MS/MS assays are prone to problems at estradiol concentration <5 pg/mL [2,9,11]. LC–MS/MS assays are not without challenges [10]. 17β-Estradiol is the active stereoisomer of estradiol whereas its epimer, 17α-estradiol has no estrogenic activity (Fig. 3). Since 17β-estradiol and 17α-estradiol are isobars, specificity of LC–MS/MS assays is dependent on chromatographic separation.

Most large reference clinical laboratories offer LC–MS/MS estradiol assays. Advantages of LC–MS/MS over immunoassays for the measurement of androgens and estrogens have been reviewed widely [10,20,30]. Pre-analytical and analytical issues that warrant consideration for developing a LC–MS/MS estradiol assay include: (a) sample preparation to extract estradiol from serum, (b) derivatization conditions (if any) [20,30], (c) chromatographic methods [17,18] and (d) mass spectrometer method (electrospray or APCI using positive or negative mode [18,31,32]). Sample preparation methods involving protein precipitation using acetonitrile [19], liquid–liquid extraction (LLE) using methylene chloride [18] and methyl tertiary butyl ether [17] and solid phase extraction have been described [17]. Although enhanced sensitivity and specificity has been observed with derivatization [18], the need for a derivatization step in steroid LC–MS/MS assays has been debated [20]. A limit of detection of 1–2 pg/mL has been reported using a non-derivatized, negative mode, electrospray ionization method [19].

We developed and validated a high sensitivity LC–MS/MS assay for estradiol (Singh and Girtman; unpublished results). The assay involves LLE of 0.5 mL serum sample with methylene chloride followed by derivatization with dansyl chloride, separation using a two-dimensional ultra-performance liquid chromatography system and analysis on an Agilent 6490 mass spectrometer using electrospray ionization on a positive mode. The combination of derivatization and two dimensional chromatography (separation on two columns (Loading: Agilent Zorbax XDB-C18; 2.1 × 30 mm 3.5 micron; Analytical: Agilent Poroshell 120 EC-C18; 2.1 × 50 mm 2.7 micron)) afforded improved sensitivity and precision. Fig. 4 shows a chromatographic peak in a patient sample at a 0.4 pg/mL estradiol concentration. Using this method we achieved a limit of quantitation of 0.3 pg/mL for estradiol with a chromatographic run time of 8.5 min per analytical run. Percent coefficient of variation (CV) for within run and between day imprecision observed over a concentration range of 0.23–405 pg/mL is shown in Tables 1 and 2.

4. Conclusion

Several studies and the recent Endocrine Society Position Statement have addressed the deficiencies in current immunoassays used for the quantitation of estradiol [2,8,9,12,13]. Direct immunoassays lack appropriate sensitivity at low estradiol levels (<40 pg/mL). Although a GC–MS estradiol assay is considered the gold standard, it is complex to use in a routine clinical laboratory and is not amenable to high test through put. LC–MS/MS estradiol assays offer the throughput, sensitivity and precision required for estradiol quantitation over a wide range of physiological concentrations. Patients receiving AI therapy are a subset for whom high sensitivity estradiol assays are particularly useful as treatment efficacy depends on the degree of estradiol suppression. Whereas, automated direct immunoassays will remain as the first line approach for the vast majority of clinical situations where the demand for assay sensitivity is modest (example: women undergoing ovulation induction), high sensitivity LC–MS/MS assays with LOQ between 0.1 and 0.2 pg/mL are needed for optimal clinical management of certain subset of patients like those receiving AI pharmacotherapy.

Disclosure statement

Authors have no disclosures.

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