Estradiol Serum Levels are Crucial to Understand Physiological/Clinical Setting in Both Sexes: Limits of Measurement of Low Estradiol and Evaluation of a Sensitive Immunoassay

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Abstract

Background: Measure of serum 17β-Estradiol is essential to understand physiology, development and health of reproductive processes in both sexes. Commercially immunoassays are not enough sensitive and accurate to assess low concentrations of E2. Purpose of this study was to compare a new sensitive immunoassay for estradiol measurement with respect to method current in our laboratory and to evaluate the performance of the new method.

Methods: Four pools of patient sera with E2 concentrations close to clinical decision values were prepared. To test the repeatability of new method the 5x5 protocol was used and CVs were calculated. To evaluate the performance of new method, 50 samples with E2 concentrations covering the whole measurable interval were selected and assayed. Linearity dilution, LoB (Limit of Blank) and LoD (Limit of Detection) were determined.

Results: The new assay showed good total repeatability demonstrated by low CV values, and good linear relationship with respect to current method (R=0.9926) as demonstrated by linear regression. Non-parametric regression showed for the new method a slight constant and proportional systematic error that, however, resulted not significant from difference plot analysis, with a general tendency to overestimate results for the current method. Performances of the new method resulted acceptable within the maximum admissible error derived from the literature, and a good linearity over a wide range of dilutions was showed. LoD value confirmed an amelioration in measurement of low estradiol.

Conclusion: In conclusion, sensitive assay is suitable to replace the method used in our laboratory with significant improvement in the measurement of low serum estradiol levels.

Keywords Lab methods comparison; Low estradiol concentration; Immunometric assay; Regression analysis; Limit of detection

Introduction

17β-Estradiol (E2) is the predominant steroid hormone belonging to estrogens according to the receptor type to which they bind. It is the primary female sex hormone produced mainly by ovary, but it is also secreted by the adrenal gland and placenta during pregnancy, and controls the development and maintenance of female sexual characteristics. E2 is also synthesized at low levels in males because some peripheral target tissues express the enzyme aromatase and this elicits the conversion of circulating

testosterone to E2 and androstenedione to estrone. In males, E2 plays a critical role in sexual function being that it is essential for modulating spermatogenesis, libido and erectile function. Therefore, the accurate measure of serum estradiol is essential to understand physiology, development and health of reproductive processes in both sexes as well as the cause of diseases related to estrogens.

Immunometric measurement of steroid hormones originally required solvent extraction, chromatographic separation and structurally authentic tracers to avoid interference from similar steroid cross-reactivity and matrix effects. The assay simplification, due to increasing demand for steroid hormones dosage, introduced bias and lack of specificity particularly at low steroid concentrations [1]. In clinical laboratories, E2 is mainly measured by direct (without solvent extraction) immunometric assays on highly automated instruments that are usually robust, economical and precise. This sensitivity and specificity is adequate for determination of high serum concentrations of E2 in females between menarche and menopause, especially during ovarian stimulation in medically assisted procreation procedures. When the circulating E2 concentrations are 10 to 100 fold lower as in children, men, postmenopausal women, and patients treated with aromatase inhibitors, the existing methods are much less indicated [1]. Estradiol is classified by WHO as a "type A" analyte since it is a well-defined compound. Its measurement should be independent from the used method, but performance quality for E2 assay has often been questioned [2-5]. Difficulties in analytical determination of E2 may be due to interference from similar molecules, to low concentrations in the serum of many subjects, to low analytical specificity and to possible lack of traceability of E2 standards. The original gold standard to quantify serum estradiol was the Radioimmunoassay (RIA) first described by Abrahm in 1969 [6]. This method requires initial purification achieved by organic solvent extraction and column chromatography separation of E2 before quantification. Nowadays, gas and liquid chromatography coupled with tandem mass spectrometry (GC-MS/MS and LC-MS/MS) represent the gold standard methods for E2 measurement. It has been shown that GC-MS/MS is insensitive when compared with immunoassay. It is laborious and requires expensive equipment not suitable for routine purposes [7-9], [10,11]. LC-MS/MS offers instead a sensitivity comparable to that of immunoassays, shorter run times and, despite the high cost of instrumentation, it appears suitable for measuring E2 when the concentration is too low to be measured by immunoassay [9].

Beckman Coulter recently put on the market the new Access Sensitive Estradiol assay offering improved measurement of low levels of E2, such as those typically found in men, pediatric populations and post-menopausal women. The aims of the present study are (i) The comparison between the new method Beckman Coulter Access Sensitive Estradiol assay and the current method Beckman Coulter Access Estradiol assay, (ii) The evaluation of performance of the new method in terms of repeatability and acceptability. Acceptability of method is evaluated on the basis of the maximum admissible analytical total error described in the literature (www.westgard.com).

Materials and Methods

E2 Measurement by Access Estradiol and Access Sensitive Estradiol assays

Estradiol measurements were performed by using Access Estradiol assay and Access Sensitive Estradiol assay on Beckman Coulter UniCel DxI 600 automated platforms (Beckman Coulter Diagnostics, Brea, CA, USA) according to the manufacturer's instructions.

Repeatability of Access Sensitive Estradiol

To test the repeatability of the new method, four pools of patient sera with E2 concentrations close to clinical decision values were prepared. Level 1 at E2 concentration about 20 pg/mL, Level 2 at E2 concentration about 300-400 pg/mL, Level 3 at E2 concentration about 1000-2000 pg/mL, and Level 4 at E2 concentration about 3500-4500 pg/mL. For each level once a day for 5 days, 5 independent replicates of the same sample were analyzed by using the Access Sensitive Estradiol assay (5x5 protocol) on one automated platform. In each session the analytical quality was assessed by using control charts, and results, expressed as pg/mL, were statistically analyzed as described below.

Performance of Access Sensitive Estradiol

To evaluate the performance of the new method, 65 serum samples previously measured with Access Estradiol assay, and having E2 concentrations covering the whole measurable interval and the range of values clinically observable, were selected. Samples in duplicate were assayed over a period of 9 days by using the Access Sensitive Estradiol assay and the obtained results were subjected to graphical and statistical analysis as described below.

The dilutional linearity of samples is important to validate specificity and accuracy of a method. A serum sample with an E2 concentration included in standard curve working range (2721.94 pg/mL) was selected to perform the linearity dilution test. The sample was analyzed undiluted and diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512 and the linearity dilution curve was constructed. For each sample, the % change in concentration from the previous dilution was determined. Dilutional linearity was considered acceptable if the concentration of the analyte corrected for the dilution factor varied no more than 80%-120% between doubling dilutions [12].

Statistical and graphical analysis

Statistical analysis of results was carried out using the MedCalc statistical software for biomedical research (www.medcalc.org). For the repeatability test the daily mean, standard deviation and variance were calculated. General mean, variance within days, variance between days and total variance within the laboratory were also calculated in order to obtain total repeatability expressed as coefficient of variation (CV%).

To assess the performance of the new method, the following statistical and graphical analysis of data were performed: linear regression analysis and Pearson correlation coefficient determination, non-parametric regression analysis of Passing-Bablok with a Confidence Interval (CI) of 95% [13,14], Bland-Altman analysis with a CI of 95% [15] and Method Decision Chart (MEDx chart) construction [16].

Analytical sensitivity: Limit of Blank (LoB) and Limit of Detection (LoD)

To detect analytical sensibility of the new method, LoB and LoD were determined. LoB is the highest apparent analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested: $LoB = mean_{blank} + 1.645 (SD_{blank})$. LoD is the lowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible. LoD is determined by utilising both the measured LoB and test replicates of a sample known to contain a low concentration of analyte: $LoD = LoB + 1.645 (SD_{low\ concentration\ sample})$ [17,18].

To determine LoB and LoD two different lots of Access Sensitive Estradiol assay were used (Lot1 and Lot2) and only one Beckman Coulter UniCel DxI 600 automated platform (Beckman Coulter Diagnostics, Chaska, MN, USA).

Four blank samples were analysed to determine LoB: S1 (calibrator 0 Lot1), S2 (calibrator 0 Lot2), S3 (calibrator 0 Lot3) and S4 (pool of negative patient sera with E2 <15 pg/mL).

Four pools of patient serum with low E2 concentration were analysed to determine LoD: S5 (pool of patient sera with E2 15-20 pg/mL), S6 (pool of patient sera with E2 15-20 pg/mL), S7 (pool of patient sera with E2 15-20 pg/mL) and S8 (pool of patient sera with E2 15-20 pg/mL). For each sample were measured 5 replicates, for 2 reagent lots, for 3 days, for a total of 30 measurements.

Ethics

Having performed the research on pre-existing serum samples, anonymized and deidentified prior to start the study, referred for routine E2 determination, no institutional review board approval was necessary. Authors had not access to any private health information from the patients involved. However, informed consent was obtained by all subjects enrolled in the study. The research has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Results

Repeatability of Access Sensitive Estradiol

Since the new method was already validated by the manufacturer following Approved Guideline from Clinical and Laboratory Standard Institute [19-24], a rapid protocol was carried out to verify what was declared. Following suggestions in recent literature we adopted 5x5 protocol in order to obtain a more realistic estimation of repeatability [25].

The 5×5 protocol indicated a very good total repeatability as shown by the low values of coefficient of variation ranging from

Table 1: Coefficient of variation (CV) expressed as percentage for different concentrations of E2.
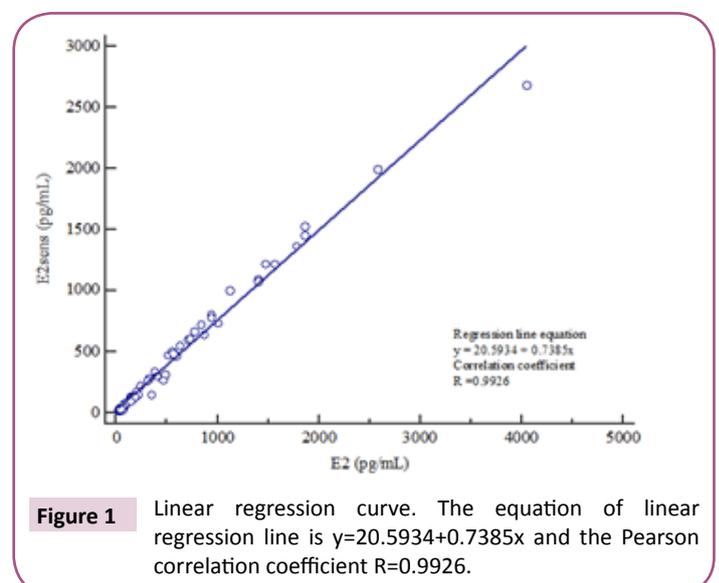
E2 concentration pg/mL	CV %
Level 1	7.3
Level 2	1.5
Level 3	2.8
Level 4	3.6

1.5% to 7.3% for the different levels of E2 concentration (Table 1). The highest CV value, whilst still within 10% of variability, was observed, as expected, at the lower E2 concentration levels.

Performance of Access Sensitive Estradiol

From linear regression analysis the compared methods showed a good linear relationship with a Pearson correlation coefficient R of 0.9926 (Figure 1). However, simple linear regression assumes that the current method is free of error (reference method) and that the error of the new method is normally distributed and constant at all studied concentrations, but these assumptions are rarely met in practice. For this reason alternative regression models are recommended, such as the non-parametric method [13,14]. In fact, non-parametric Passing-Bablok regression (Figure 2) highlighted a slight constant and proportional systematic error for the new method (value 0 not included in CI 95% for intercept and value 1 not included in CI 95% for slope). Cusum test for linearity, only testing the applicability of the Passing-Bablok method, indicated no significant deviation from linearity (p=0.83), and the residual plot representing the distribution of differences around the fitted regression line, showed that residuals are randomly distributed above and below the regression line, with a greater dispersion at E2 concentrations higher than about 300 pg/mL measured with the method current (Supplemental Figure 1A-B).

Another useful graphical analysis is that of Bland-Altman, especially when the measuring interval is large as in the case of E2 [15]. The diagram allows the highlighting of systematic differences between the two methods and defines them in a
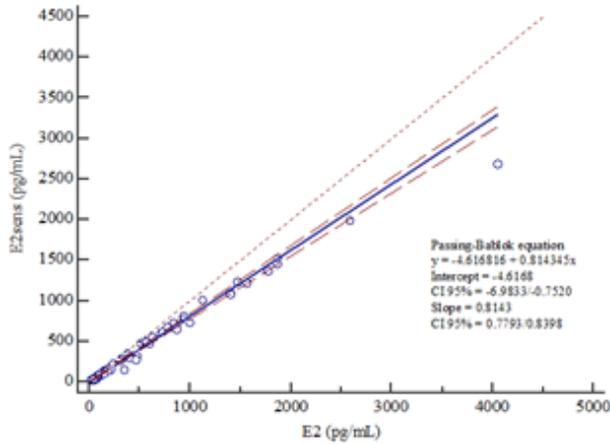

Figure 1 Linear regression curve. The equation of linear regression line is y=20.5934+0.7385x and the Pearson correlation coefficient R=0.9926.

Figure 2 Passing-Bablok regression curve. The equation of Passing-Bablok regression line is y=-4.616816+0.814345x with a CI 95% for intercept of -6.9833/-0.7520 and a CI 95% for slope of 0.7793/0.8398.
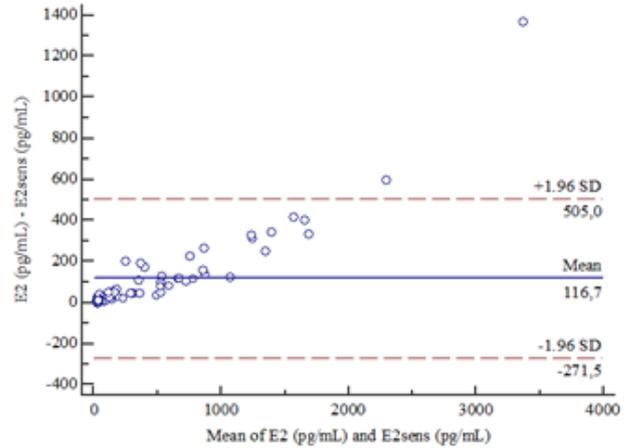

Figure 3 Bland-Altman plot. The differences between observation pairs are plotted against their mean and the average of the differences (bias) and its 95% confidence limit lines are drawn on the same plot.

quantitative way. The Bland-Altman graphical analysis in which the zero value is included in CI 95% confirmed a slight systematic but not significant error (Figure 3). Moreover we also observed for the current method a general tendency to overestimate results with respect to the new method that is more evident at high concentrations of analyte (Figure 3).

Acceptability of the new method, based on the maximum acceptable error obtained from literature, was determined by the method evaluation decision chart, MEDx chart [16] for judging method performance. The imprecision and the systematic deviation or bias are plotted respectively on the abscissas and on the ordinates, representing as a point the performance of the new method. To construct the MEDx chart, the value corresponding to the maximum acceptable error for E2 (26.86%), obtained from Desirable Biological Variation Database specifications at www. westgard.com, was reported on the y axis, while on the x axis were reported five values (maximum error divided for 2, 3, 4, 5 and 6). This corresponds to acceptability or quality criteria, allowing the division of the graph into six areas (from right to left in the graph: unacceptable, poor, marginal, good, excellent, world-class). The coordinates of the new method's performance point uses the imprecision calculated from the repeatability test for the abscissa, and the bias obtained using the equation of the non-parametric regression line applied to a certain level of E2 concentration for the ordinate.

The new method for Level 1 of concentration showed a performance located in the area of unacceptable quality, whereas for Level 2 performance, this was located in the area of good-excellent quality, for Level 3 in the area of marginal-poor quality and for Level 4 in the area of marginal quality (Figure 4A-C). Although for the Level 1 sample, the new assay is classified by MEDx chart as unacceptable (Figure 4A), a CV of 7.3% (Table 1) was obtained at this concentration (20 pg/mL), which represents a very good imprecision. What is more, for the current Access Estradiol assay the manufacturer declared a CV of 21% for
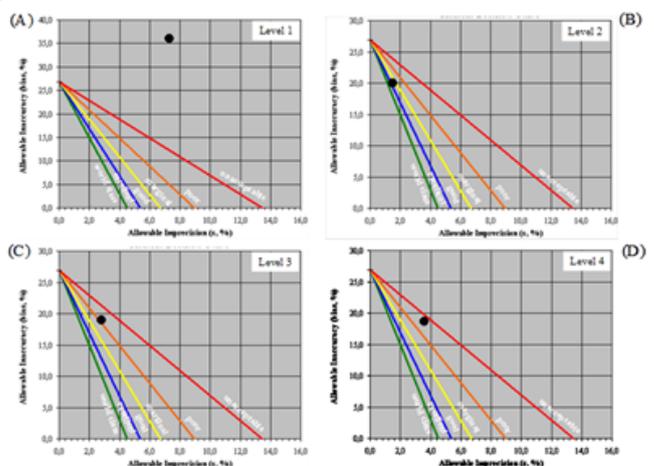

Figure 4 MEDx chart for different levels of E2 concentration. (A) Level 1 with performance in the area of unacceptable quality; (B) Level 2 with performance in the area of good-excellent quality; (C) Level 3 with performance in the area of marginal-poor quality; (D) Level 4 with performance in the area of marginal quality.

concentration of 50 pg/mL, and in our lab for concentration 17.8 pg/mL of EQA Immunocheck (Qualimedlab s.r.l., www. qualimedlab.it), a CV value of about 50% was obtained, documenting a very high imprecision for low E2 levels measured with the current method.

In the dilution linearity test, up to 1:128 dilution, the obtained concentration values corrected for dilution factor were between 89% and 117% of the whole sample. On the other hand, further dilutions of the sample provided results greater than 120% or not determinable when compared to the non-diluted sample (Figure 5). The new method, showing a good linearity over a wide range of dilutions, also provides flexibility and reliability to measure samples with different concentrations of E2, in particular samples with high levels of analyte can be diluted
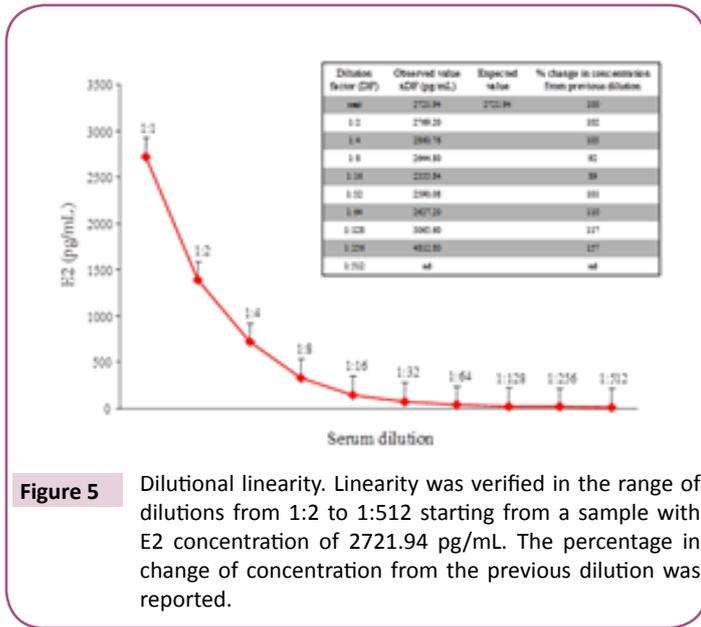
Figure 5 Dilutional linearity. Linearity was verified in the range of dilutions from 1:2 to 1:512 starting from a sample with E2 concentration of 2721.94 pg/mL. The percentage in change of concentration from the previous dilution was reported.

Figure 6 LoB and LoD calculation. (A) The LoB value was assessed by using the formula described in Materials and Methods section. Results are expressed as pg/mL; (B) The LoD value was assessed by using the formula described in Materials and Methods section. Results are expressed as pg/mL.

several times to ensure that its values fall within the standard curve range without affecting accuracy and precision.

Analytical sensitivity of Access Sensitive Estradiol

The LoB value, determined by using four blank samples as previously explained, was 8.63 pg/mL, with a range of observation from 0.00 to 13.08 pg/mL (Figure 6A). From the bar graph of frequency distribution of E2 levels expressed as Relative Luminescence Units (RLU) can be visually evaluated that data are symmetrically distributed (normal or gaussian distribution), as demonstrated by normal distribution curve superimposed over the histogram (with mean and standard deviation showed) (Supplemental Figure 2).

The LoD value, determined by using four pools with low E2 concentration, was 13.99 pg/mL, with a range of observation from 7.05 to 22.75 pg/mL (Figure 6B). Also for LoD, the bar graph of frequency distribution of E2 levels expressed as RLU, showed a normal distribution of data (Supplemental Figure 3).

The manufacturer reported for LoB a critical value ≤ 10 pg/mL E2 with a range of observed results between 5.0 and 7.5 pg/mL. For LoD the declared critical value was ≤ 15 pg/mL E2 with a range of 9.4-12.4 pg/mL. Therefore, results obtained in analytical sensitivity determination in terms of LoB and LoD were in perfect agreement with the manufacturer's declaration on the product data sheet.

In particular, the LoD value of 13.99 pg/mL calculated from our data set not only confirmed what was declared by the manufacturer, but also allowed to state that Access Sensitive Estradiol assay offers improved measurement of low levels of analyte.

Discussion

Although the determination of estradiol levels in the blood is crucial for understanding the physiological and clinical settings in both sexes, low concentrations 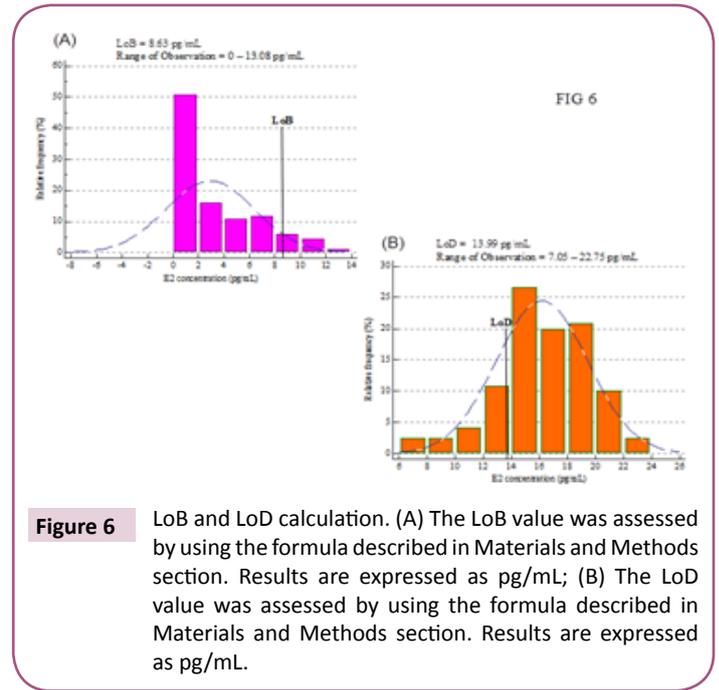of estradiol (<30 pg/mL) are difficult to measure routinely when using direct immunometric assays, including chemiluminescent and enzymatic immunoassays, without a preceding purification step [26-28]. In fact, the increasing demand for steroid hormones dosage led to laboratory assay simplification to allow the use of unextracted serum on highly automated instrumentation platforms. This introduced bias and lack of specificity mainly at low concentrations [1] such as those typically found in men, pediatric populations and post-menopausal women. As mentioned previously, GC-MS/MS and LC-MS/MS represent the two reference methods but these methods are not very suitable for routine use [7-10].

In this study we compare a new sensitive immunoassay for E2 measurement with respect to the method current in our laboratory and evaluate the performance of the new method in terms of repeatability and acceptability. The 5×5 protocol indicated a good total repeatability as demonstrated by low values of CV for the different levels of concentration. From linear regression analysis the compared methods showed a good linear relationship, however, non-parametric Passing-Bablok regression highlighted a slight constant and proportional negative bias for the new method. The Bland-Altman graphical analysis confirmed this slight systematic but not significant bias with a general tendency to overestimate results using the current method. In addition to good precision at the low E2 concentration, performances of the new method resulted acceptable within the maximum admissible error derived from the literature as demonstrated by the method decision chart (performance located in the areas of acceptability). Dilution linearity tests showed good linearity over a wide range of dilutions and, finally, the LoD value demonstrated an improvement for measurement of low estradiol concentrations when compared to the current method.

There is no doubt that the Access Sensitive Estradiol assay provides improved precision and accuracy at the low estradiol levels. For low E2 levels, literature reports elevated CV values as in the paper by Hendelsman et al. [1] where a CV of 23.7% and a

bias of 120% was reported for a concentration level of 34 pg/mL measured with LC-MS reference method.

In the human body, estradiol is metabolized to more than 100 conjugated and unconjugated metabolites and many of them may cross-react with antibodies used in immunoassays, producing an overestimation of results [29]. An extraction step in direct assays may remove potentially interfering substances, in particular water soluble cross-reacting steroids, rendering E2 results much more similar to that obtained with indirect assays including the extraction phase [27]. Direct assays have several advantages, in particular for routine use and to perform large epidemiological studies; they require less quantity of sample and are less laborious, having characteristics that conform to the high automation of the assay. However, they are less accurate for the non-specific binding of interfering molecules or for unclear matrix effects. In fact, matrix differences between serum samples and pure solutions of estradiol used to generate the standard curve in a direct immunoassay may also affect validity of results. In particular, hemolyzed and lipemic samples may interfere with the binding of antigen to antibody [29].

The difficulties encountered when measuring E2 concentration in serum samples are more or less the same as those encountered when measuring all steroid hormones, and are related to the adaptation of immunoassay to valid measurement of nonimmunogenic small molecules such as steroids [1]. Steroids should be conjugated to big immunogenic proteins to develop specific antibodies, but this allows for unwelcome cross-reactivity with structurally related molecules such as precursors, metabolites and conjugates. As previously mentioned, steroid immunoassays have been used in the original methods employing solvent extraction of samples, chromatography separation and structurally authentic tracers to remove interferences from similar molecules and matrix. The growing demand for E2 measurement, especially to monitor ovarian response to gonadotropin stimulation in the emergent practice of medically assisted procreation, has led to the marketing of highly automated immunoassays without extraction, chromatography and authentic tracers, suitable for routine purposes but much less specific and accurate. These assays are particularly aimed at measuring physiological (up to 500 pg/mL) or dangerously high (more than 2000 pg/mL) concentrations of E2. High specificity and accuracy are, however, necessary to measure low levels of E2 such as those that occur in men, postmenopausal women, children and aromatase inhibitor treated patients. To date, mass spectrometry is the reference method for measuring sex hormone levels in male and female [10,30]. Furthermore, a recombinant cell ultrasensitive estrogen bioassay which correlates well with GC-MS/MS data was described [31]. Though modern immunoassays for estradiol are reasonably well suited for the diagnosis and management of infertility (despite imprecision and differences between methods), the very low concentrations crucial in non-reproductive tissues are still difficult to measure [7,8]. With low-end precision (LoD ≤ 15 pg/mL) and state-of-the-art sensitivity (LoQ ≤ 19 pg/mL), the new Access Sensitive Estradiol assay may help laboratories to deliver more accurate results for patients seeking answers to reproductive health questions, and its use is particularly indicated for the measurement of very low levels of estradiol to assess particular clinical conditions such as inborn errors of sex-steroid metabolism, disorders of puberty, estrogen deficiency in men, therapeutic drug monitoring during low-dose female hormone replacement therapy and antiestrogen treatment.

In conclusion, the new method has a very good total repeatability as shown by low CV values and, even in the presence of a minimum proportional systematic bias compared to the current method, is acceptable within the maximum admissible error obtained from literature. It also demonstrated a good linearity over a wide range of dilutions and is flexible and reliable for analyzing samples with high levels of analyte after dilution. Finally, from LoD value it is possible to state that this assay also offers improved measurement of low levels of serum estradiol.

Conflict of Interest

CM, MRS, PV, MT, PA have none to declare.

MCA is employed by Beckman Coulter which is the manufacturer of assays used in this work.

Author Contribution

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Competing Interest

The authors have declared that no competing interests exist.

Data Availability

All data are fully available without restriction.

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