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

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 to accurately assess low concentrations of E2. Purpose of this study was to compare a new 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 of LoB (Limit of Blank) and LoD (Limit of Detection) was 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 error that, however, resulted not ant from a reference 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 concentrations was showed. LoD value confirmed an amelioration of low estradiol.

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

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

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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 circular...
testosterone to E2 and androstenedione to estrone. In males, E2 plays a role in sexual development and is the precursor to androgen hormones. Therefore, the accurate measure of serum estradiol is essential for understanding 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 specific tracers to avoid interference from similar steroid cross-reactions. Matrix effects also affected performance quality for E2 assays on highly automated instruments that are usually robust, economical and precise. This method requires 10 to 100 fold lower as in children, men, postmenopausal women, and patients treated with aromatase inhibitors, the use of tracers is much less indicated [1]. Estradiol is measured by immunoassay as a “type A” analyte since it is a well-characterized compound. Its measurement should be independent from the used method, but performance quality for E2 assay has been questioned [2-5]. In analogy, determination of E2 may be due to interference from similar molecules, to low concentrations in the serum of many subjects, to low antibody and to possible lack of traceability of E2 standards. The original gold standard to quantify estradiol was the Radioimmunoassay (RIA) described by Abraham in 1969 [6]. This method requires achieved by organic solvent extraction and column chromatography separation of E2 from quan [7,8]. 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 e when compared with immunoassay. It is laborious and requires expensive equipment not suitable for research purposes [7-9], [10,11]. LC-MS/MS is instead a highly comparable to that of immunoassays, shorter run 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 e Estradiol assay offering improved measurement of low levels of E2, such as those typically found in men, pediatric populations and pre-menopausal women. The aims of the present study are (i) The comparison between the new method Beckman Coulter Access e 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 analyte 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 e 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 concentration 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 e Estradiol assay (5x5 protocol) on one automated platform. In each session the analytic 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 observed, were selected. Samples in duplicate were assayed over a period of 9 days by using the Access e Estradiol assay and the obtained results were subjected to graphical and statistical analysis as described below.

The linearity of samples is important to validate and accuracy of a method. A serum sample with an E2 concentration included in standard curve working range (272.94 pg/mL) was selected to perform the linearity 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 % change in concentration from the previous was determined. Dilution factor varied no more than 80%-120% between doubling.

Statistical and graphical analysis

Statistical analysis of results was carried out using the MedCalc 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 variance.
To assess the performance of the new method, the following sta and graphical analysis of data were performed: linear regression analysis and Pearson correlation coefficient determination of non-parametric regression analysis of Passing-Bablok with a Con Interval (CI) of 95% [13,14], Bland-Altman analysis with a CI of 95% [15] and Method Decision Chart (MEDX chart) cons

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

To detect analyte sensitivity 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 (SDblank). LoD is the lowest analyte concentration likely to be reliably determined from the LoB and at which detection is feasible. LoD is determined by both the measured LoB and test replicates of a sample known to contain a low concentration of analyte: LoD=LoB+1.645 (SD low concentra) [17,18].

To determine LoB and LoD two reagent lots of Access Sensitive Estradiol assay were used (Lot1 and Lot2) and only one Beckman Coulter UniCel DxI 600 automated platform (Beckman Coulter Diagnostics, 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 sera with E2 <15 pg/mL). Each sample was measured 5 replicates, for 2 reagent lots, for 3 days, for a total of 30 measurements.

### Ethics

Having performed the research on pre-existent serum samples, anonymized and deiden prior to the study, referred for review board approval was necessary. Authors had not access to any private health information from the participants 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 (Declara 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 repeatability [25].

The 5x5 protocol indicated a very good total repeatability as shown by the low values of coefficient of variation ranging from 1.5% to 7.3% for the low levels of E2 concentrations (Table 1). The highest CV value, whilst still within 10% of variability, was observed, as expected, at the lower E2 concentration level.

![Linear regression curve](image)

**Table 1:** Coefficient of variance (CV) expressed as percentage for different levels of E2 concentration.

| E2 concentration pg/mL | CV % |
|------------------------|------|
| Level 1                | 7.3  |
| Level 2                | 1.5  |
| Level 3                | 2.8  |
| Level 4                | 3.6  |

Performance of Access Sensitive Estradiol

From linear regression analysis the compared methods showed a good linear relationship with a Pearson coefficient 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 tests the applicability of the Passing-Bablok method, indicated no ant deviation from linearity (p=0.83), and the residual plot represents the distribution of errors around the ed regression line, showed that residuals are randomly distributed above and below the regression line, with a greater dispersion at E2 concentration 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 highlights system differences between the two methods and de them in a
The Bland-Altman graphical analysis in which the zero value is included in CI 95% confirmed a slight systematic but not constant 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 (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 represented on the abscissas and on the ordinates, representing 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 Variance Database 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: 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 or 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 was by the 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 high precision. What is more, for the current Access Estradiol assay the manufacturer declared a CV of 21% for concentrations of 50 pg/mL, and in our lab for concentrations of 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 linearity test, up to 1:128 the obtained values corrected for factor were between 89% and 117% of the whole sample. On the other hand, further 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 concentrations also provides stability and reliability to measure samples with low and high levels of analyte can be diluted.
Several methods are available for understanding the physiological and clinical significance of estradiol levels in the blood. However, none of the current methods are perfectly suited to the measurement of low estradiol levels. Traditional methods such as radioimmunoassays (RIA) and competitive binding assays can provide improved precision and accuracy at the low estradiol levels. For low estradiol levels, literature reports elevated CV values as high as 100%.

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 0.00 to 13.08 pg/mL (Figure 6A). From the bar graph of frequency distribution of LoB values expressed as Rela Temp Luminescence Units (RLU), it can be visually evaluated that data are symmetrically distributed (normal or gaussian distribution). As demonstrated by normal distribution, in the histogram, the LoB value was ≤ 10 pg/mL, with a range of observed results between 5.0 and 7.5 pg/mL. Therefore, results obtained in analyte are in agreement with the manufacturer's declaration of LoB and LoD were in perfect agreement with the manufacturer's declaration.

The manufacturer reported for LoB a value ≤ 10 pg/mL E2 with a range of observed results between 5.0 and 7.5 pg/mL. LoD for LoB the declared value was ≤ 5 pg/mL E2, with a range of 9.4-12.4 pg/mL. Therefore, results obtained in analyte determined in terms of LoB and LoD were in perfect agreement with the manufacturer's declaration.

In this study we compared a new immunoassay for estradiol measurement with respect to the method currently in our laboratory and post-menopausal women. As men and women have different levels of estradiol levels in the blood, it is crucial to ensure that its values fall within the standard curve range without a significant change of concentration. Therefore, results using the current method. In this study, the LoB value of 13.99 pg/mL, with a range of observed results between 5.0 and 7.5 pg/mL. Therefore, results obtained in analyte determined in terms of LoB and LoD were in perfect agreement with the manufacturer's declaration.

Discussion

Although the determination of estradiol levels in the blood is crucial for understanding the physiological and clinical significance of estradiol levels in both sexes, low concentrations of estradiol (<30 pg/mL) are less frequently detected. This is mainly due to the limitations of traditional methods such as RIA and competitive binding assays. However, Access Sensitive Estradiol assay provides improved precision and accuracy at the low estradiol concentration levels. For low estradiol levels, literature reports elevated CV values as high as 100%.

In this study we compared a new immunoassay for estradiol measurement with respect to the method currently 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 lowest levels of concentration. From linear regression analysis the compared methods showed a good linear relation however, non-parametric Passing-Bablok regression highlighted a slight constant and proportional bias for the new method. The Bland-Altman graphical analysis confirmed this slight systematic bias but not a significant random error derived from the literature as demonstrated by the method decision chart (performance located in the areas of acceptability).

Linearity tests showed good linearity over a wide range of concentrations and, the LoD value demonstrated an improvement for measurement of low estradiol concentration levels 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 estradiol levels, literature reports elevated CV values as high as 100%.
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 an antibody in immunoassays, producing an overestimation of results [29]. An extra step in direct assays may remove potential interfering substances, in water soluble cross-react steroids, rendering E2 results much more similar to that obtained with indirect assays including the extraphase [27]. Direct assays have several advantages, in far fewer use and to perform large epidemiological studies; they require less quantity of sample and are less laborious, having characteristics that conform to the high autonomy of the assay. However, they are less accurate for the non-binding of interfering molecules or for unclear matrix effects. In fact, matrix differences between serum samples and pure ones 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 an antibody to an estradiol.

The difficulties encountered when measuring E2 concentrations in serum samples are more or less the same as those encountered when measuring all steroid hormones, and are related to the adaptability of immunoassay to valid measurement of nonimmunogenic small molecules such as steroids [1]. Steroids should be conjugated to big immunogenic proteins to develop highly sensitive assays, but this allows for unwelcome cross-react with structurally related molecules such as precursors, metabolites and conjugates. As previously men steroid immunoassays have been used in the original methods employing solvent extractions, chromatography separations and structurally authored 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 postmenopausal or medically assisted procreation, has led to the marking of highly automated immunoassays without extractions, chromatography and authored tracers, suitable for routine purposes but much less accurate. These assays are aimed at measuring physiological (up to 500 pg/mL) or dangerously high (more than 2000 pg/mL) concentrations of E2. High sensitivity 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 pa tients. To date, mass spectrometry is the reference method for measuring sex hormone levels in male and female [10,30]. Furthermore, a recombinant cell utlizing estradiol biosassay 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 conditions (despite imprecision and between methods), the very low concentration crucial in non-reproductive health questions and its use is indicated for the measurement of very low levels of estradiol to assess 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 an estrogen 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 proportion of matrix 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 concentrations and is suitable and reliable for analyzing samples with high levels of analyte estrogen. Finally, from LoD value it is possible to state that this assay also improves measurement of low levels of serum estradiol.

Conflict of Interest
CM, MRS, PV, MT, PA have none to declare.

Author Contribution
All the authors have accepted responsibility for the content and editorial decisions in this manuscript and approved submission.

Competing Interest
The authors have declared that no competing interests exist.

Data Availability
All data are fully available without restrictions.

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