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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Received: May 20, 2019; Accepted: July 22, 2019; Published: July 29, 2019

Abstract

**Background:** Measure of serum 17β-Estradiol is essential to understand physiology, development and health of reproductive processes in both sexes. Commercially available immunoassays are not enough sensitive and accurate to 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 (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 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 E2 was showed. LoD value confirmed an improvement of low estradiol.

**Conclusion:** In conclusion, the new 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 concentrations; 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 circu...
testosterone to E2 and androstenedione to estrone. In males, E2 plays a key role in sexual differentiation; hence, it is an essential component of male development, including in the reproductive system. It is also involved in the regulation of sexual desire and libido.  

Repeatability 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 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 (272.194 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 obtained results were subjected to graphical and statistical analysis as described below.

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Statistical and graphical analysis  
Stata 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 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 to determine non-parametric regression analysis of Passing-Bablok with a Conformed Interval (CI) of 95% [13,14], Bland-Altman analysis with a CI of 95% [15] and Method Decision Chart (MEDx chart) concepts.

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=meanblank +1.645 (SDblank). LoD is the lowest analyte concentration likely to be reliably detected 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 (SDlow concentra) [17,18].

To determine LoB and LoD, two blank lots of Access Sensitive Estradiol assays were used (Lot1 and Lot2) and only one Beckman Coulter UniCel DxI 600 automated platform (Beckman Coulter Diagnostics a, 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 sera 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, five replicates were measured 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 the study, referred for rE2 determina no ins review board approval was necessary. Authors had not access to any private health information from the participants. 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 the recent literature we adopted 5×5 protocol in order to obtain a more realistic repeatability [25].

The 5×5 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 different levels of E2 concentration (Table 1). The highest CV value, whilst s within 10% of variability, was observed, as expected, at the lower E2 concentration level.

Performance of Access Sensitive Estradiol

From linear regression analysis, the compared methods showed a good linear relationship with a Pearson correlation 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 concentration but these assumptions are rarely met in practice. For this reason, alternate 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 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 test 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 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 identification of systematic errors between the two methods and demonstrates them in a

<table>
<thead>
<tr>
<th>E2 concentration pg/mL</th>
<th>CV %</th>
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<tbody>
<tr>
<td>Level 1</td>
<td>7.3</td>
</tr>
<tr>
<td>Level 2</td>
<td>1.5</td>
</tr>
<tr>
<td>Level 3</td>
<td>2.8</td>
</tr>
<tr>
<td>Level 4</td>
<td>3.6</td>
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Figure 1: Linear regression curve. The equation of linear regression line is y=20.5934+0.7385x and the Pearson correlation coefficient is R²=0.9926.
The Bland-Altman graphical analysis in which the zero value is included in CI 95% confirmed a slight systematic but not ant 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 bias are plotted 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 Variability 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 good imprecision. What is more, for the current Access Estradiol assay the manufacturer declared a CV of 21% for concentra 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 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 also provides flexibility and reliability to measure samples with different concentrations of E2, in samples with high levels of analyte can be diluted.
several to ensure that its values fall within the standard curve range without a lack of acuity 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 observed values from 0.00 to 13.08 pg/mL (Figure 6A). From the bar graph of frequency distribution of E2 levels expressed as Relu Luminescence Units (RLU) can be visually evaluated that data are symmetrically distributed (normal or Gaussian distribution) as demonstrated by normal 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 Relu showed a normal distribution.

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

In addition, 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 provided 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 significance of both sexes, low concentrations of estradiol (<30 pg/mL) are

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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-r steroid e ion systems, rendering E2 results much more similar to that obtained with indirect assays including the extraplate [27]. Direct assays have several advantages, in far less laborious, having characteristics that conform to the high automaticity of the assay. However, they are less accurate for the non-specific binding of interfering molecules or for unclear matrix effects. In fact, matrix interferences between serum samples and pure non-human estradiol used to generate the standard curve in a direct immunoassay may also affect validity of results. In those situations, hemolized and lipemic samples may interfere with the binding of an anti-estradiol antibody. The cross-reactivity encountered when measuring E2 concentrations in serum samples is more or less the same as those encountered when measuring all steroid hormones, and are related to the adapta of immunoassay to validate measurement of nonimmunogenic small molecules such as steroids [1]. Steroids should be conjugated to big immunogenic proteins to develop highly specific and pure assays, but this allows for unwelcome cross-reactivities with structurally related molecules such as precursors, metabolites and conjugates. As previously mentioned, immunoassays have been used in the original methods employing solvent extractions, chromatography separations 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, has led to the market of highly automated immunoassays without extractions, chromatography and authentic tracers, suitable for rapid 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 patients. To date, mass spectrometry is the reference method for measuring sex hormone levels in male and female [10,30]. Furthermore, a recombinant cell ultrastrogen 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 interferences between methods), the very low concentrations of estradiol in serum and the cross-reaction of highly specific antibodies lead to an overestimation of results [7,8]. With lower end precision (LoD ≤ 15 pg/mL) and state-of-the-art technology (LoQ ≤ 19 pg/mL), the new Access estradiol assay may help laboratories to deliver more accurate results for patients seeking answers to reproducible 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 anovular 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 propofol 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 reliable and reliable for analyzing samples with high levels of analyte estradiol. Finally, from LoD value it is possible to state that this assay also provides improved 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 have approved submission.

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

Data Availability
All data are fully available without restriction.

References


