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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 essen to understand physiology, development and health of repr e processes in both sexes. Commercially immunoassays are not enough e and accurate to assess low concentra of E2. Purpose of this study was to compare a new e 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 pa t sera with E2 concentra 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 concentra covering the whole measurable interval were selected and assayed. Linearity LoB (Limit of Blank) and LoD (Limit of Det ere determined.

Results: The new assay showed good total repeatability demonstrated by low CV values, and good linear rela ship 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 pr systema error that, however, resulted not ant from erence plot analysis, with a general tendency to overes te 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 was showed. LoD value con a ement of low estradiol.

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

Keywords Lab methods comparison; Low estradiol concentra Immunometric assay; Regression analysis; Limit of det

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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 characteris E2 is also synthesized at low levels in males because some peripheral target express the enzyme aromatase and this elicits the conversion of circula

testosterone to E2 and androstenedione to estrone. In males, E2 plays a al role in sexual being that it is essen for modula spermatogenesis, libido and er on. Therefore, the accurate measure of serum estradiol is essen to understand physiology, development and health of repr ve processes in both sexes as well as the cause of diseases related to estrogens.

Immunometric measurement of steroid hormones originally required solvent extr , chromatographic separa and structurally authen tracers to avoid interference from similar steroid cross-r and matrix e ects. The assay a due to increasing demand for steroid hormones dosage, introduced bias and lack of at low steroid concentra [1]. In clinical laboratories, E2 is mainly measured by direct (without solvent extr immunometric assays on highly automated instruments that are usually robust, economical and precise. This and is adequate for determina of high serum concentra of E2 in females between menarche and menopause, especially during ovarian s in medically assisted procrea procedures. When the circula E2 concentra are 10 to 100 fold lower as in children, men, postmenopausal women, and pa ts treated with aromatase inhibitors, the exis methods are much less indicated [1]. Estradiol is by WHO as a "type A" analyte since it is a well-de compound. Its measurement should be independent from the used method, but performance quality for E2 assay has en been ques [2-5]. in analy al determina of E2 may be due to interference from similar molecules, to low concentra in the serum of many subjects, to low analy al and to possible lack of traceability of E2 standards. The original gold standard to quan y serum estradiol was the Radioimmunoassay (RIA) st described by Abrahm in 1969 [6]. This method requires a achieved by organic solvent extr and column chromatography separa of E2 before quan a 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 r purposes [7-9], [10,11]. LC-MS/MS ers instead a vity comparable to that of immunoassays, shorter run and, despite the high cost of instrumenta it appears suitable for measuring E2 when the concentra is too low to be measured by immunoassay [9].

Beckman Coulter recently put on the market the new Access e Estradiol assay ering improved measurement of low levels of E2, such as those typically found in men, pediatric popula and post-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 evalua of performance of the new method in terms of repeatability and acceptability. Acceptability of method is evaluated on the basis of the maximum admissible analy al 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 pla orms (Beckman Coulter Diagnos Brea, CA, USA) according to the manufacturer's ins

Repeatability of Access Sensitive Estradiol

To test the repeatability of the new method, four pools of pa t sera with E2 concentra close to clinical decision values were prepared. Level 1 at E2 concentra about 20 pg/mL, Level 2 at E2 concentra about 300-400 pg/mL, Level 3 at E2 concentra about 1000-2000 pg/mL, and Level 4 at E2 concentra 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 pla orm. In each session the analy al quality was assessed by using control charts, and results, expressed as pg/mL, were sta ally 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 concentra 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 sta al analysis as described below.

The linearity of samples is important to validate and accuracy of a method. A serum sample with an E2 concentra included in standard curve working range (2721.94 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 linearity n curve was constructed. For each sample, the % change in concentra from the previous was determined. Di linearity was considered acceptable if the concentra of the analyte corrected for the factor varied no more than 80%-120% between doubling

Statistical and graphical analysis

Sta al analysis of results was carried out using the MedCalc sta al are for biomedical research (www.medcalc.org). For the repeatability test the daily mean, standard devia 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 coe t of varia

To assess the performance of the new method, the following sta al and graphical analysis of data were performed: linear regression analysis and Pearson correla coe t determina 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 analy al sensibility of the new method, LoB and LoD were determined. LoB is the highest apparent analyte concentra 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 concentra likely to be reliably dis ished from the LoB and at which det is feasible. LoD is determined by both the measured LoB and test replicates of a sample known to contain a low concentra of analyte: LoD=LoB+1.645 (SD$_{low\ concentra}$) [17,18].

To determine LoB and LoD two erent lots of Access e Estradiol assay were used (Lot1 and Lot2) and only one Beckman Coulter UniCel DxI 600 automated pla orm (Beckman Coulter Diagnos 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 nega e pa t sera with E2 <15 pg/mL).

Four pools of pa t serum with low E2 concentra were analysed to determine LoD: S5 (pool of pa t sera with E2 15-20 pg/mL), S6 (pool of pa t sera with E2 15-20 pg/mL), S7 (pool of pa t sera with E2 15-20 pg/mL) and S8 (pool of pa t 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-exis serum samples, anonymized and deiden prior to start the study, referred for r E2 determina no ins review board approval was necessary. Authors had not access to any private health informa from the pa ts 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 Associa (Declara 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 Ins e [19-24], a rapid protocol was carried out to verify what was declared. Following sugges in recent literature we adopted 5x5 protocol in order to obtain a more realis epeatability [25].

The 5×5 protocol indicated a very good total repeatability as shown by the low values of coe t of varia ranging from 1.5% to 7.3% for the erent levels of E2 concentra (Table 1). The highest CV value, whilst s within 10% of variability, was observed, as expected, at the lower E2 concentra vels.

Table 1: Coe t of varia (CV) expressed as percentage for erent concentra

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 rela with a Pearson correla coe t 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 concentra but these assump are rarely met in pr For this reason alterna e 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 pr systema 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 tes the applicability of the Passing-Bablok method, indicated no ant devia from linearity (p=0.83), and the residual plot represen the dis of erences around the ed regression line, showed that residuals are randomly distributed above and below the regression line, with a greater dispersion at E2 concentra 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 highligh of systema erences between the two methods and de them in a

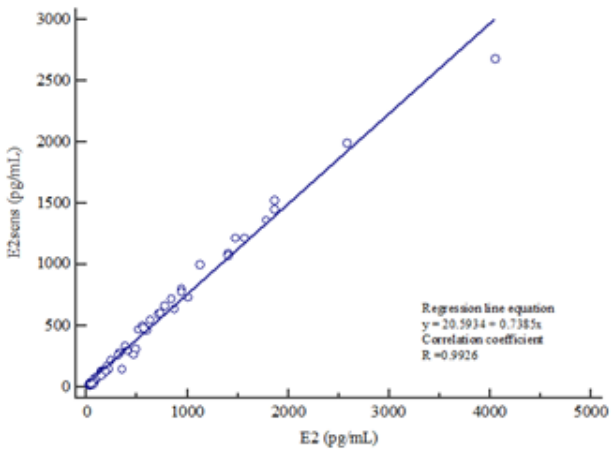

Figure 1 Linear regression curve. The equa of linear regression line is y=20.5934+0.7385x and the Pearson correla oe t R=0.9926.

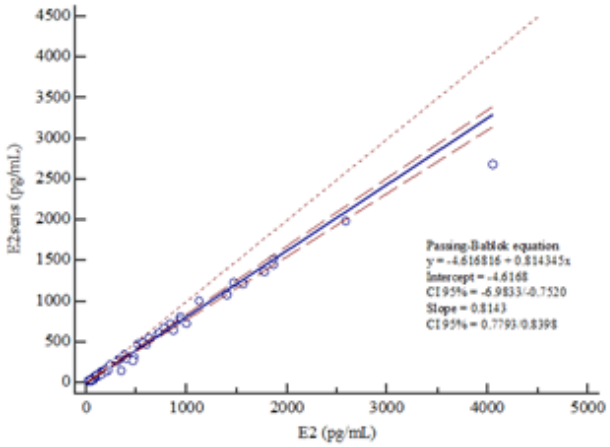

Figure 2 Passing-Bablok regression curve. The equa of Passing-Bablok regression line is y=-4.616816+0.814345x with a CI 95% for intercept of -6.9833/-0.7520 and a CI 95% for slope of 0.7793/0.8398.

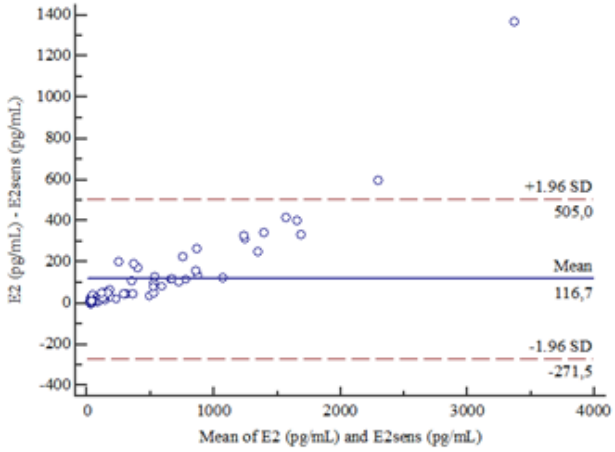

Figure 3 Bland-Altman plot. The erences between observa pairs are ed against their mean and the average of the erences (bias) and its 95% con limit lines are drawn on the same plot.

quan a e way. The Bland-Altman graphical analysis in which the zero value is included in CI 95% con a slight systema but not ant error (Figure 3). Moreover we also observed for the current method a general tendency to overes te results with respect to the new method that is more evident at high concentra te (Figure 3).

Acceptability of the new method, based on the maximum acceptable error obtained from literature, was determined by the method evalua decision chart, MEDx chart [16] for judging method performance. The imprecision and the systema devia or bias are ed r ely on the abscissas and on the ordinates, represen 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 Varia Database a at www.westgard.com, was reported on the y axis, while on the x axis were reported e 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 le 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 equa of the non-parametric regression line applied to a certain level of E2 concentra or the ordinate.

The new method for Level 1 of concentra 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 by MEDx chart as unacceptable (Figure 4A), a CV of 7.3% (Table 1) was obtained at this concentra (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

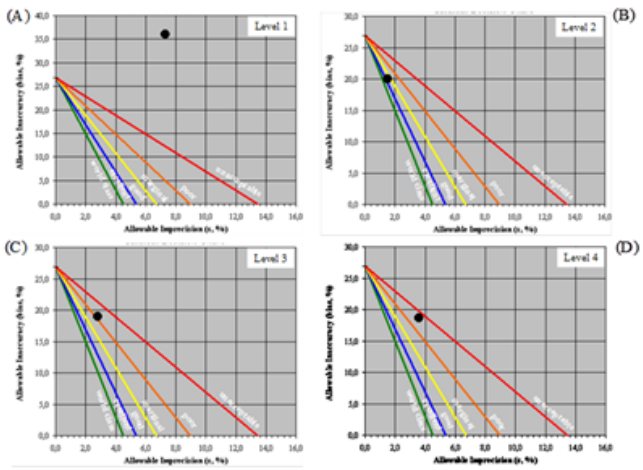

Figure 4 MEDx chart for di erent levels of E2 concentra (A) Level 1 with performance in the area of unacceptable quality; (B) Level 2 with performance in the area of good-excellent quality; (C) Level 3 with performance in the area of marginal-poor quality; (D) Level 4 with performance in the area of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

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

In the linearity test, up to 1:128 the obtained concentra 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 xibility and reliability to measure samples with erent concentra of E2, in samples with high levels of analyte can be diluted

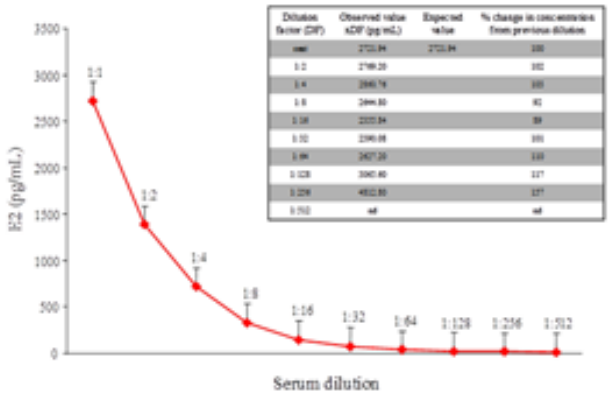

Figure 5 linearity. Linearity was v in the range of from 1:2 to 1:512 st from a sample with E2 concentra of 2721.94 pg/mL. The percentage in change of concentra from the previous was reported.

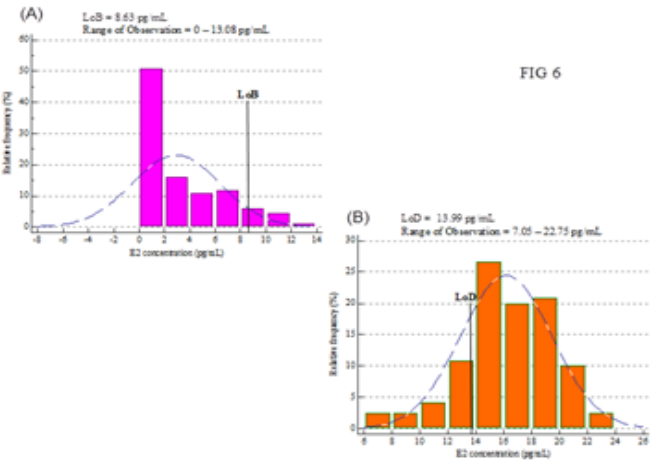

Figure 6 LoB and LoD calcula (A) The LoB value was assessed by using the formula described in Materials and Methods Results are expressed as pg/mL; (B) The LoD value was assessed by using the formula described in Materials and Methods Results are expressed as pg/mL.

several to ensure that its values fall within the standard curve range without a acy 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 observa from 0.00 to 13.08 pg/mL (Figure 6A). From the bar graph of frequency dis of E2 levels expressed as Rela e Luminescence Units (RLU) can be visually evaluated that data are symmetrically distributed (normal or gaussian dis), as demonstrated by normal dis curve superimposed over the histogram (with mean and standard devia showed) (Supplemental Figure 2).

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

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

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

Discussion

Although the determina of estradiol levels in the blood is crucial for understanding the physiological and clinical se in both sexes, low concentra of estradiol (<30 pg/mL) are lt to measure r when using direct immunometric assays, including chemiluminescent and enzyma immunoassays, without a preceding a step [26-28]. In fact, the increasing demand for steroid hormones dosage led to laboratory assay a to allow the use of unextracted serum on highly automated instrumenta pla orms. This introduced bias and lack of mainly at low concentra [1] such as those typically found in men, pediatric popula and post-menopausal women. As men previously, GC-MS/MS and LC-MS/MS represent the two reference methods but these methods are not very suitable for r

In this study we compare a new e 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 erent levels of concentra From linear regression analysis the compared methods showed a good linear rela however, non-parametric Passing-Bablok regression highlighted a slight constant and pr nega e bias for the new method. The Bland-Altman graphical analysis con this slight systema but not ant bias with a general tendency to overes te results using the current method. In to good precision at the low E2 concentra 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). linearity tests showed good linearity over a wide range of ns and, , the LoD value demonstrated an improvement for measurement of low estradiol concentra s when compared to the current method.

There is no doubt that the Access e 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 concentra 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 used in immunoassays, producing an overes of results [29]. An extr step in direct assays may remove poten interfering substances, in water soluble cross-r steroids, rendering E2 results much more similar to that obtained with indirect assays including the extr phase [27]. Direct assays have several advantages, in for r use and to perform large epidemiological studies; they require less quan of sample and are less laborious, having characteris that conform to the high automa of the assay. However, they are less accurate for the non binding of interfering molecules or for unclear matrix e ects. In fact, matrix erences between serum samples and pure ons of estradiol used to generate the standard curve in a direct immunoassay may also a ect validity of results. In , hemolyzed and lipemic samples may interfere with the binding of an en to an

The encountered when measuring E2 concentra in serum samples are more or less the same as those encountered when measuring all steroid hormones, and are related to the adapta of immunoassay to valid measurement of nonimmunogenic small molecules such as steroids [1]. Steroids should be conjugated to big immunogenic proteins to develop an ies, but this allows for unwelcome cross-r with structurally related molecules such as precursors, metabolites and conjugates. As previously men steroid immunoassays have been used in the original methods employing solvent extr of samples, chromatography separa and structurally authen tracers to remove interferences from similar molecules and matrix. The growing demand for E2 measurement, especially to monitor ovarian response to gonadotropin s in the emergent pr of medically assisted procrea has led to the marke of highly automated immunoassays without extr chromatography and authen tracers, suitable for r purposes but much less and accurate. These assays are aimed at measuring physiological (up to 500 pg/mL) or dangerously high (more than 2000 pg/mL) concentra of E2. High y 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 ts. To date, mass spectrometry is the reference method for measuring sex hormone levels in male and female [10,30]. Furthermore, a recombinant cell ultr e 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 inf (despite imprecision and erences between methods), the very low concentra crucial in non-repr e are s to measure [7,8]. With low-end precision (LoD ≤ 15 pg/mL) and state-of-the-art y (LoQ ≤ 19 pg/mL), the new Access e Estradiol assay may help laboratories to deliver more accurate results for pa ts seeking answers to repr e health ques and its use is indicated for the measurement of very low levels of estradiol to assess clinical c such as inborn errors of sex-steroid metabolism, disorders of puberty, estrogen de in men, ther drug monitoring during low-dose female hormone replacement therapy and an trogen 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 propo systema 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 and is xible and reliable for analyzing samples with high levels of analyte a er . Finally, from LoD value it is possible to state that this assay also ers 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 en e content ed manuscript and approved submission.

Competing Interest

The authors have declared that no compe terests exist.

Data Availability

All data are fully available without res

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