Performance of Direct Estradiol Immunoassays with Human Male Serum Samples

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BACKGROUND: Steroid immunoassays originally required solvent extraction, chromatography, and structurally authentic tracers to avoid interference from steroid cross-reactivity and matrix effects. The demand for steroid assays has driven assay simplification, bypassing this triplet of validity criteria to allow use of unextracted serum, which has introduced bias and nonspecificity at low steroid concentrations. We aimed to evaluate the performance of commercial direct estradiol (E2) immunoassays relative to the reference method of LC-MS and compared serum E2 measurements from each assay with biomarkers of estrogen action.

METHODS: We measured serum E2 in duplicate using 5 commercial direct immunoassays and LC-MS in a nested cohort of 101 healthy, asymptomatic men >40 years old from the Healthy Man Study. For each immunoassay, we evaluated the detectability and distribution of serum E2 measurements, CV, and bias (relative to LC-MS) by Passing–Bablok regression and deviance plots.

RESULTS: Three assays detected E2 in all samples, whereas E2 was detected in only 53% and 72% of samples by 2 other assays. All 5 assays had positive biases, ranging from 6% to 74%, throughout their ranges. CVs were lower with 4 immunoassays than with LC-MS. LC-MS, but none of the direct immunoassays, correlated with serum testosterone and sex steroid–binding globulin.

CONCLUSIONS: The positive bias of direct E2 immunoassays throughout their working range reflects the nonspecific effects of steroid cross-reactivity and/or matrix interference arising from the violation of the triplet validity criteria for steroid immunoassay.

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Accurate measurement of serum estradiol (E2)5 is crucial to understanding reproductive development, physiology, and health as well as the origins of estrogen-dependent diseases. Whereas the androgens testosterone (T) and dihydrotestosterone exert dominant influence on male reproductive physiology and pathology, aromatization of T to E2 has been recognized as an important local regulatory hormonal mechanism for certain tissues in men, notably brain (1) and bone (2). Consequently, the indications for serum E2 measurements in men include clinical evaluation of gynecomastia; steroid-secretting testicular, adrenal, or hepatic tumors; or genetic aromatase excess, androgen insensitivity, or Peutz–Jeghers syndrome. In addition, serum E2 measurement may be useful in monitoring anti-estrogen or aromatase inhibitor treatment for pubertal delay (with or without short stature), obesity, or male infertility and a threshold of circulating E2 for maintenance of bone density in older men. Further clinical indications for serum E2 measurement in men may have been overlooked due to the limitations of E2 immunoassays for the low concentrations in male blood samples, notably in conditions of possible estrogen deficiency states or apparent estrogen excess, including hormone-sensitive cancers (3). Thus, measurement of serum E2 is as important for understanding numerous developmental and pathophysiological processes in men as it is in women. Yet although modern commercial E2 immunoassays, which have been optimized for unextracted human serum (“direct”), may be adequate for measuring the high blood E2 concentrations in women between men-

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5 Nonstandard abbreviations: E2, estradiol; T, testosterone; ISO, International Organization for Standardization; RCPAQAP, Royal College of Pathologists of Australasia Quality Assurance Programs; CRM, certified reference material; LH, luteinizing hormone; FSH, follicle-stimulating hormone; SHBG, sex hormone–binding globulin; BMI, body mass index; BSA, body surface area; IQR, interquartile range.
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arche and menopause, and especially during ovarian stimulation, circulating E₂ concentrations are 10- to 100-fold lower in children, men, and postmenopausal or aromatase inhibitor–treated women and are challenging to measure. Awareness of problems with validity (analytical specificity, accuracy) of direct immunoassays, which was identified >25 years ago (4, 5), grew over the last decade following the recognition of limitations of T immunoassays, particularly at low circulating concentrations (6–8). Direct T immunoassays display assay-specific bias and analytical nonspecificity, most notably at low blood T concentrations in children, women, and androgen-deficient men (6–10). Therefore, aiming to measure the expected (picomolar) serum E₂ concentrations that render T immunoassays unreliable makes it unsurprising that direct E₂ immunoassays lack accuracy and validity for measurements of serum E₂ in not only men but also children (11) as well as postmenopausal (4, 12–14) or aromatase inhibitor–treated (15) women who have comparably low blood E₂ concentrations (16). Comparisons of direct E₂ immunoassays with mass spectrometry–based reference methods in men have previously been confined to single commercial E₂ immunoassays (17–19), whereas other studies focused on in–house E₂ immunoassays (15, 20) or studied only women (4, 12, 13). Here we used a reference panel of sera to calibrate and compare the performance of widely used commercial direct E₂ immunoassays relative to the reference LC-MS method using a reference panel of healthy older men (8).

Materials and Methods

SAMPLES
A reference panel of sera was formed from a nested cohort of participants in the Healthy Man Study (21) who were >40 years old and reported excellent or very good health with no health symptoms including sexual dysfunction or gynecomastia. Previously unthawed aliquots of serum (n = 101) collected during the study were distributed frozen to 5 laboratories, all holding accreditation to International Organization for Standardization (ISO) 15189 and participating in the national RCPAQAP (Royal College of Pathologists of Australasia Quality Assurance Programs) quality assurance program. The laboratories, selected to represent different commercial direct E₂ immunoassays, each ran the samples in duplicate in each assay.

ASSAYS
The 5 commercial direct E₂ immunoassays were (A) Siemens ADVIA Enhanced Estradiol assay run on Centaur analyzer; (B) Siemens IMMULITE 2000 Estradiol assay run on IMMULITE 2000 analyzer; (C) Abbott ARCHITECT System Estradiol assay run on ARCHITECT analyzer; (D) Roche cobas Estradiol II assay run on Roche E170 analyzer; and (E) Beckman Coulter Access Estradiol assay run on Dxi800 analyzer (these 5 immunoassays are listed in this article as A–E, respectively). All 5 methods provide reference intervals for E₂ in males, indicating that measurement of male sera is included in the intended use of the assays, and 4 of the 5 methods claim traceability to GC-MS. The samples were also measured by an LC-MS E₂ assay (22). To validate the serum E₂ measurements in the LC-MS assay, 3 certified reference materials (CRMs) for serum E₂ (CRM 576, CRM 577, and CRM 578) obtained from the European Commission’s Institute for Reference Materials and Measurements (23) were run in duplicate on consecutive days in the LC-MS assay, including 1 set run together with the samples in this study. Serum T was measured in the same sample by the same LC-MS method (22) and serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), and sex hormone–binding globulin (SHBG) by immunoassays as reported previously (21).

DATA ANALYSIS
For each immunoassay, the distribution of values was determined by nonparametric methods, distributional deviation from gaussian by Shapiro–Wilk statistic, and bias (relative to MS) by Passing–Bablok regression and illustrated by deviance (modified Bland–Altman) and mountain plots (24) by use of NCSS, MedCalc, and Sigmaplot software. Samples with undetectable serum E₂ values were deleted from analysis of that immunoassay. We calculated body mass index (BMI) as weight (in kg) divided by the square of height (in meters) and body surface area (BSA) in m² by the Gehan–George formula (25). We calculated CV for an assay as the mean of the CVs for the 2 replicates for each sample without adjustment for serum E₂ concentration. Correlation was performed by linear least-squares analysis with a Bonferroni adjustment for multiple comparisons. Results are presented as mean and SE if gaussian, or as median and interquartile range (IQR) (25th, 75th centiles) otherwise.

Results

The 101 participants had a median (IQR) age of 54 (48–62) years and BMI 25.9 (23.9–28.6) kg/m². The group had a mean (SE) height 176 (0.7) cm, weight 81.2 (1.1) kg, and BSA 2.00 (0.01) m².

In terms of the assigned target serum E₂ concentrations, the CRM samples had a median accuracy of 100% [101% (6%)] at low concentrations (nominal certified concentration 31 pg/mL), 93% [93% (3%)] at medium concentrations (188 pg/mL), and 89% [89%...
at high concentrations (365 pg/mL) without any significant between-day difference ($P > 0.8$).

The overall analysis of serum E$_2$ measurements (repeated-measures ANOVA) showed highly significant difference between assays ($F_{5995} = 8.84$, $P < 10^{-6}$) and between men ($F_{95,995} = 9.10$, $P < 10^{-6}$) but not for intraassay replication ($F_{1995} = 0.0$, $P = 1.0$). Three immunoassays detected serum E$_2$ in all samples, whereas E$_2$ was detected in only 53% of samples in assay B and 72% in assay E. Table 1 lists the nonparametric centiles of distribution and pooled within-assay CVs for each immunoassay, and the immunoassays are compared in Table 2. Fig. 1 shows the overall distribution of serum E$_2$ concentrations displayed as notched box plots. For each immunoassay, the serum E$_2$ measurements had a nongaussian distribution in the natural scale (Table 1), and these were normalized by a logarithmic transformation for each assay except assay B, which had numerous undetectable samples (data not shown).

The bias parameters for each immunoassay relative to LC-MS are displayed as Passing–Bablok regressions (Fig. 2) and regression estimates (Table 1) as well as mountain plots (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue3) and deviance plots (Fig. 3). The bias of each method was upward (positive) ranging from 12% to 53% (at mean) and 11% to 74% (at 25th centile), 6% to 55% (at median), and 11% to 48% (at 75th centile). Residual SD in the Passing–Bablok regressions were similar for 4 immunoassays and LC-MS but were significantly higher for assay B, which reflected its lower analytical sensitivity, lack of linearity, and greater deviation from regression slope of 1.0. The CVs were lower for 4 assays (A, B, C, and D) relative to LC-MS but higher in 1 (E).

To evaluate biological correlates of each assay, serum E$_2$ concentrations from the 5 E$_2$ immunoassays and LC-MS E$_2$ assay were correlated by linear regression with weight, BMI, and reproductive hormone

<table>
<thead>
<tr>
<th>Table 1. Serum E$_2$ distribution centiles and Passing–Bablok analysis.$^a$</th>
<th>LC-MS</th>
<th>A$^b$</th>
<th>B$^c$</th>
<th>C$^d$</th>
<th>D$^e$</th>
<th>E$^f$</th>
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<tr>
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<td>363</td>
<td>285</td>
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<td>185</td>
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<td>118</td>
<td>87</td>
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<td>98</td>
<td>100</td>
</tr>
<tr>
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<td>79</td>
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<td>76</td>
<td>73</td>
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<td>100</td>
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<td>100</td>
<td>100</td>
<td>72</td>
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<td>Gaussian$^g$</td>
<td>0.95$^h$</td>
<td>0.86$^h$</td>
<td>0.56$^h$</td>
<td>0.92$^h$</td>
<td>0.81$^h$</td>
<td>0.67$^h$</td>
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<tr>
<td>Mean (SD)</td>
<td>83 (28)</td>
<td>122 (30)</td>
<td>102 (43)</td>
<td>127 (30)</td>
<td>104 (37)</td>
<td>106 (35)</td>
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<td>CV, %$^i$</td>
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<td>Reference</td>
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<td>17</td>
<td>45$^k$</td>
<td>15$^k$</td>
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<td>√</td>
<td>X</td>
<td>√</td>
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</table>

$^a$ Serum E$_2$ concentrations are in pmol/L. To convert to pg/mL, divide by 3.67.

$^b$ A, Siemens ADVIA.

$^c$ B, Siemens IMMULITE 2000.

$^d$ C, Abbott ARCHITECT.

$^e$ D, Roche cobas.

$^f$ E, Beckman Coulter Access.

$^g$ Shapiro-Wilks W.

$^h$ Significant ($P < 0.05$) deviation from gaussian distribution.

$^i$ Mean coefficient of variation of duplicate measurements.

$^j$ Correlation with LC-MS.

$^k$ Significant ($P < 0.05$) difference from slope = 1.0 or intercept = 0.0.
variables (serum LH, FSH, T, SHBG) (Table 3). Serum E$_2$ correlated with serum LH and SHBG in the LC-MS assay but not with either gonadotropin in any immunoassays. Assay B results correlated negatively with weight and BMI, but this was not observed with any other assay. None of the assays generated serum E$_2$ results that correlated with age, height, or bone density at hip or spine (in either absolute bone density or T scores; data not shown).

**Discussion**

In this study, we used a defined reference panel (8) comprising 101 serum samples from healthy older men, a nested cohort of the Healthy Man Study (21), to calibrate the performance of 5 commercial direct E$_2$ immunoassays as has been done previously for serum T immunoassays (8). On the basis of measurements of this reference panel, each immunoassay deviated significantly from the reference LC-MS method throughout the working range, with the magnitude of positive (upward) bias varying between assays from 6% to 74%. As the variability seen here is based on single versions of each assay, a significantly greater variability might be expected when interlaboratory variability is included (26). It is possible that the immunoassays are able to separate concentrations within reference intervals from clearly increased serum E$_2$ in men; however, method-dependent reference intervals would be required. This study did not address that question.

Our findings extend previous reports comparing direct E$_2$ immunoassays with MS-based methods in men but where only a single commercial immunoassay (17–19) or in-house immunoassays (15, 20) were examined. Our results are also consistent with the findings in postmenopausal or aromatase inhibitor–treated women with comparably low circulating E$_2$ concentrations (4, 12, 13, 27). The strong, positive
The correlation of the LC-MS assay with serum T and SHBG was not matched by any immunoassay. The failure of serum E2 concentration to correlate with BMI, proposed as a biological validity criterion for serum E2 measurements in women \(28\), may reflect the biological differences in the range of circulating E2 concentrations between sexes. The striking negative correlation of weight or BMI with serum E2 concentration by assay B, but no other assay, may reflect the unknown cross-reacting steroids, responsible for the overestimation of serum E2 measurements and poor correlation with LC-MS, which may correlate inversely with weight or obesity. Because this assay produced fewer detectable values covering a narrower range of results, the weaker correlation with LC-MS is an expected finding.

The reasons for these discrepancies are related to the origins of steroid immunoassays, which were developed in 1969 \(29\) but delayed for a decade after the invention of peptide immunoassay because additional steps were required to adapt immunoassay methodology to valid measurement of nonimmunogenic small molecules such as steroids. Developing steroid-specific antibodies required conjugating steroids as haptens to larger immunogenic carrier proteins via small multivalent reactive bridge compounds. However, this made steroid antibodies that were epitope "blind" to the conjugation site, allowing for undesirable cross-reactivity with structurally related steroids (e.g., structurally related steroid precursors, metabolites, and conjugates produced by phase II metabolism). The original in-house steroid immunoassays developed in the 1970s used solvent extraction, chromatography, and structurally authentic \(\text{H}^3, \text{C}^{14}\) tracers, a triplet of validity criteria for steroid immunoassay that removed structurally related steroids as well as nonspecific matrix interference in the immunoassay reactions. In the ensuing decades, E2 immunoassays were commercialized primarily to monitor ovarian responses to gonadotropin stimulation, where excessive serum E2 response was a risk indicator for dangerous overdosage effects.

Fig. 2. Passing–Bablok regression analysis of serum E2 concentrations in LC-MS (MS) and 5 commercial E2 immunoassays (A–E) in 101 sera from healthy, asymptomatic men over the age of 40 years. The x axis is the reference method (LC-MS), and the y axis represents the measurements from 1 of 5 commercial E2 immunoassays (A–E). The diagonal line represents the line of identity, the heavier line the Passing–Bablok regression line, and the outer dotted lines the 95% confidence limits.

A, Siemens ADVIA.
C, Abbott ARCHITECT.
D, Roche cobas.
E, Beckman Coulter Access.
such as high-order multiple pregnancies and/or life-threatening ovarian hyperstimulation syndrome. However, making a quantal distinction between dangerously high E2 concentrations (>2000 pmol/L) and typical premenopausal concentrations (200 – 800 pmol/L) did not require quantitative accuracy at physiological E2 concentrations. Subsequently, ultrasound monitoring of follicular growth has reduced the importance of this application of serum E2 immunoassays. The subsequent growing demand for steroid immunoassays in clinical practice and research resulted in assay simplification to adapt steroids into semi-automated multiplex platforms and 1-plate/tube kit formats, preferred by routine pathology and research labs, respectively. This simplification eliminated preassay purification steps (extraction, chromatography) as well as β-scintillation counting, with the latter forcing the replacement of structurally authentic steroid tracers with bulky conjugated steroids, which allowed for more

| Table 3. Correlation between serum E2 concentrations measured by 5 immunoassays and LC-MS assay and weight, BMI, and reproductive hormones. |
|---------------------------------|--------|--------|--------|--------|--------|
| **MS** | **A** | **B** | **C** | **D** | **E** |
| Weight | -0.14 | -0.17 | -0.38 | -0.20 | -0.21 | -0.21 |
| BMI    | -0.18 | -0.09 | -0.41 | -0.27 | -0.18 | -0.24 |
| LH     | 0.08  | 0.08  | 0.20  | 0.13  | 0.19  | -0.07 |
| FSH    | -0.06 | -0.03 | 0.03  | 0.02  | 0.03  | -0.15 |
| T      | 0.45  | 0.26  | 0.07  | 0.12  | 0.25  | 0.06  |
| SHBG   | 0.34  | 0.17  | 0.24  | 0.05  | 0.26  | 0.03  |

* A, Siemens ADVIA.
* B, Siemens IMMULITE 2000.
* C, Abbott ARCHITECT.
* D, Roche cobas.
* E, Beckman Coulter Access.

* P < 0.001 for a nominal P < 0.05 with Bonferroni adjustment for 36 pair-wise comparisons.

* P < 0.001 for a nominal P < 0.05 with Bonferroni adjustment for 36 pair-wise comparisons.

Fig. 3. Deviance (modified Bland–Altman) analysis of deviations of serum E2 concentrations measured in 5 commercial E2 immunoassays (A–E) from the reference method (LC-MS) in 101 sera from healthy, asymptomatic men over the age of 40 years.

The x axis is the reference method (LC-MS), and the y axis represents the deviations of measurements from 1 of 5 commercial E2 immunoassays (A–E) from the reference method. The horizontal solid line represents the mean deviation, and the horizontal dotted lines represent the 95% confidence limits of the deviations.

A, Siemens ADVIA.
C, Abbott ARCHITECT.
D, Roche cobas.
E, Beckman Coulter Access.
convenient nonradioactive assay readouts. However, eliminating the triplet of validity criteria (preassay extraction and chromatography, authentic tracers) sacrificed the specificity of E₂ immunoassays and revealed the vulnerabilities of direct steroid immunoassay to artifacts from steroid cross-reactivity and matrix interference. Although the original validated steroid immunoassays are now confined to a few long-established laboratories, there is evidence that some but not all the limitations of direct E₂ immunoassays can be overcome by preassay solvent extraction (12, 28). However, since our study did not include any classical (indirect) estradiol immunoassays, it is not clear whether meeting the original triplet validity criteria is sufficient to overcome all limitations of direct E₂ immunoassays, and it remains possible that suboptimal antibody specificity could also contribute to the nonspecificity of steroid immunoassays.

The analytical sensitivity of 3 direct E₂ immunoassays was adequate to detect serum E₂ in all serum samples from healthy older men. This reflects generational improvement over less analytically sensitive previous E₂ immunoassays (13). Replicate imprecision was better with most platform E₂ immunoassays owing to the use of automated rather than manual pipetting; however, the most accurate direct E₂ immunoassay had replicate imprecision inferior to that of LC-MS, a long-recognized limitation of E₂ immunoassays (26). This presumably reflects the worse imprecision of immunoassays at the low end of their working range. On the other hand, the CV for the LC-MS assay in this study based on 2 replicates was higher than when a larger number of QC samples was pooled (22), although this does not exclude effects resulting from differences in internal standard:analyte ratios (30) or other deviations from ideal internal standard performance (31).

Although assay-dependent bias and analytical nonspecificity of direct E₂ immunoassays is established (11, 12, 14, 15, 27, 32–35), direct E₂ assays are often used in research involving tissue extracts and nonhuman serum samples, where their validity is doubtful (36, 37).

Overall, these findings indicate that direct E₂ immunoassays are suboptimal for use with human male serum where either absolute measurement is required, such as in clinical practice or research that hinges on interpreting E₂ measurements in single serum samples, or even in more self-contained research studies where small differences in measurements may be decisive, such as analytical epidemiology (16, 38, 39). These limitations underestimate the problems with direct E₂ immunoassays over time, since commercial kit or platform formats are subject to changes in proprietary components, notably (but not only) new antibodies, that have to be replaced. Hence direct E₂ immunoassays exhibit not only assay-dependent bias but are subject to unpredictable variability over time, all of which detract from assay performance and stability. By contrast, the advent of lower-cost, benchtop MS equipment that retains reference concentration specificity but now features analytical sensitivity matching the best steroid immunoassays renders MS-based E₂ assays more widely accessible. Although MS-based E₂ assays provide a durable chemical analysis free from methodology-dependent bias of immunoassays, like all biochemical measurements, MS-based assays may be subject to different features such as those involving matrix effects, chromatography, and differences in monitored transitions, all of which require rigorous standardization and ongoing quality control (16, 28, 40), an essential but challenging process that remains to be completed.

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