Analytical performance of Bayer Immuno 1 estradiol and progesterone assays

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We evaluated the analytical performance of new estradiol and progesterone assays performed on the Bayer Immuno 1 system. Within-run and between-day CVs for estradiol at concentrations of 116.8–6645.8 pmol/L were ≤6.4% and for 5.54–103.95 nmol/L progesterone were ≤7.7%, thus meeting published analytical goals. The detection limits (2 SDs from mean of zero calibrator) were 27.1 pmol/L for estradiol (n = 72 over 20 days) and 0.51 nmol/L for progesterone (n = 47 over 20 days). The assays were linear to 9766 pmol/L and 113.0 nmol/L, respectively. Estradiol results agreed well with the Diagnostic Products Corporation (DPC) assays, except for serum samples from patients receiving estrogen replacement therapy; results for these samples agreed closely with the DPC estradiol-6 assay. The progesterone assay agreed closely with the DPC assay, except for samples from uremic patients. Reference values were estimated by the study of 29 women throughout the menstrual cycle with 15 samples per subject. We concluded that both assays demonstrate suitable precision, linearity, and intermethod agreement to allow their use in the clinical laboratory.

The determination of estradiol and progesterone concentrations in serum is useful for the evaluation of fertility and menstrual problems in women. Serial measurements of estradiol allow the monitoring of follicular growth and predict the day of ovulation during a regular menstrual cycle [1] and ovarian stimulation therapy [2]. Serum estradiol has been shown to correlate with the diameter of the leading follicle [1, 3]. A relation between serum estradiol and the number of preovulatory follicles, oocytes, and embryos has also been found [4]. The measurement of serum estradiol is used to prevent ovarian overstimulation syndrome [5].

Serum estradiol assays are used for the evaluation of gynecomastia or feminization states caused by estrogen-secreting tumors that occur rarely in both males and females of all ages [6, 7]. Measurement of this analyte is also useful for the evaluation of precocious puberty and sexual maturation in females [8]. Monitoring of estradiol concentration for hormonal replacement therapy is sometimes practiced but has limited use, as other estrogen metabolites are predominant [9, 10].

Assessment of progesterone is important for monitoring ovulation and luteal function and has been proposed as a marker for pregnancy outcome [11]. The diagnostic value of progesterone is in the detection of ovulation and the functional evaluation of the corpus luteum. It is also useful for monitoring patient ovulation during induction therapy [11, 12]. Progesterone assays can also be used for the surveillance of gestational trophoblastic neoplasia [13] and the prediction of success of in vitro fertilization [14, 15].

Considering the clinical applications of estradiol and progesterone measurements, the assays measuring these analytes need a high sensitivity, an excellent precision, and preferably a wide analytical range.

The Bayer Immuno 1 system is an automated random access immunoassay analyzer. The estradiol and progesterone assays performed on this analyzer are based on the magnetic separation–sequential competitive immunoassay principle. These assays involve a conjugate of analyte and alkaline phosphatase (ALP) that competes with the sample analyte for the binding sites on an anti-analyte antibody conjugated to fluorescein isothiocyanate (FITC). Anti-FITC antibodies coupled to magnetic particles allow separation of the immune complexes.

In this study, we evaluated the precision, linearity, and minimum detectable concentration of the Immuno 1 es-

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4 Nonstandard abbreviations: ALP, alkaline phosphatase; FITC, fluorescein isothiocyanate; FSH, follitropin; LH, luteinizing hormone; pNPP, p-nitrophenyl phosphate.
Immuno 1 system and progesterone assays. These methods were compared with the Diagnostic Products Corp. (DPC) RIAs. The method comparison study included various categories of serum samples that could interfere with these assays. We also determined the reference ranges for the Immuno 1 estradiol, progesterone, follitropin (FSH), and lutropin (LH) assays throughout the menstrual cycle.

**Materials and Methods**

** IMMUNO 1 SYSTEM**

The Bayer (Business Group Diagnostics) Immuno 1 system is a fully automated immunoassay analyzer that can analyze in batch, random, or stat modes. This analyzer is composed of a reagent, a substrate, a sample, a buffer and immunomagnetic particle, and a reaction compartment. The Immuno 1 system also includes a workstation for system programming.

**IMMUNO 1 ASSAYS**

The Immuno 1 estradiol and progesterone assays are magnetic separation–sequential competitive immunoassays that involve an anti-analyte antibody–FITC conjugate and the analyte labeled with ALP. The estradiol assay involves a rabbit polyclonal antibody and the progesterone assay involves a monoclonal antibody. The Immuno 1 assays for FSH and LH are magnetic separation sandwich immunoassays that involve an anti-analyte antibody–FITC conjugate and a second anti-analyte antibody conjugated to ALP. The Immuno 1 FSH and LH assays were previously evaluated.

The reagent boats (Bayer) for these analytes contain reagents for 100 tests. All the assay steps are automated and performed at 37 °C. For the estradiol and progesterone assays, the samples are incubated with the anti-analyte antibody–FITC conjugate. After 15 min, the analyte–ALP conjugate is added and incubated for an additional 5 min. For the FSH and LH assays, the samples are incubated simultaneously for 20 min with an anti-analyte antibody–FITC conjugate and an anti-analyte antibody–ALP conjugate.

In all assays, the immune complexes formed are incubated for 8 min with magnetic particles coated with anti-FITC antibody. The immune complexes bound to the particles are then washed and incubated with p-nitrophényl phosphate substrate (pNPP). The absorbance is measured at 405 nm every 30 s for 5 min. The pNPP hydrolysis produces a color inversely proportional to the analyte concentration for the competitive assays and directly proportional for the sandwich assays. The time required to obtain the first sample result is 38 min and 30 additional seconds for each subsequent result.

Six concentrations of calibrators are used for the assay calibrations. The Bayer SETpoint calibrators are supplied in solution for estradiol and must be reconstituted before use for progesterone, FSH, and LH. Common calibrators are supplied for FSH and LH. Each analyte is measured in duplicate for the calibrations. The SETpoint calibrators are stable for at least 30 days at 4 °C when opened. The ranges for the calibration curves are: 0–127 nmol/L for progesterone, 0–11,030 pmol/L for estradiol, 0–150 IU/L for FSH, and 0–200 IU/L for LH.

**DPC ESTRADIOL, ESTRADIOL-6, AND PROGESTERONE ASSAYS**

The following DPC Coat-a-Count assays were used for the method comparison studies: progesterone, new progesterone, estradiol, and estradiol-6 assays. DPC changed the antibody used in their progesterone assay during the course of this study. We compared the Immuno 1 progesterone assay with both kits, which we call “new” and “old” DPC progesterone assays.

The procedure is the same for all methods. Seven calibrators are tested with each assay. The calibrators, three Lyphochek immunoassay controls (lot 93 000; Bio-Rad Labs.), and serum samples are incubated in anti-analyte antibody-coated tubes in the presence of 125I-labeled progesterone or 125I-labeled estradiol according to the assay. The tubes are incubated at room temperature for 3 h, the supernatants decanted, and the remaining radioactivity in each tube counted for 1 min.

**Precision Studies**

The precision of the Immuno 1 estradiol and progesterone assays was evaluated according to the NCCLS protocol EP5-T2 [17]. Three Bayer TESTpoint ligand controls, three human serum pools at clinically important estradiol or progesterone concentrations (Bayer), and the six respective TESTpoint calibrators were analyzed in duplicate over 20 days.

**Minimum Detectable Concentration**

The minimum detectable concentrations of the Immuno 1 estradiol and progesterone assays were evaluated with the respective Bayer TESTpoint calibrator 1 (0 nmol/L progesterone or 0 pmol/L estradiol) during the precision study. These values were obtained by calculating, from the calibration curve equations, the concentrations corresponding to the means of the reaction rates minus two total SDs. According to recent guidelines, these concentrations should correspond to the means of the reaction rates minus three total SDs; these values were also calculated.

**Dilution Linearity Studies**

Six serum samples with high concentrations of analyte were diluted with the respective TESTpoint calibrator 1 at 0%, 20%, 25%, 33%, 50%, and 100% of serum. Each dilution was tested in duplicate. The statistical evaluations were made by linear regression analysis.

**Method Comparison Studies**

We used serum samples with increased bilirubin (up to 304 μmol/L), creatinine (up to 1544 μmol/L), rheumatoid factor (>320 kIU/L), immunoglobulins (IgG up to 34.5 mg/dL), and other substances.
g/L or IgA up to 58.5 g/L), triglycerides (up to 19.8
mmol/L), and hemolysis (up to 12 g/L hemoglobin), and
serum samples from postmenopausal women, postmeno-
pausal women taking estrogens with or without proges-
tins, healthy men, diabetics, hemodialysis patients, preg-
nant women, and women receiving ovarian stimulation
treatment for infertility. The remaining serum samples
tested were chosen by their progesterone or estradiol
concentration to cover the assay analytical range.

The Immuno 1 estradiol assay was compared with the
DPC estradiol assay by using 176 samples. The samples
that showed the greatest differences between the two
assays were also analyzed by the DPC estradiol-6 assay
(n = 61). The Immuno 1 progesterone assay has been
compared with the old and new DPC progesterone assays
by using 185 and 48 samples, respectively. The serum
samples were tested by the Immuno 1 assays and by the
comparative methods (in single analyses) on the same
day. The statistical evaluations were made by linear
regression and bias plot analyses.

REFERENCE VALUES
Serum from 29 menstrual-cycling women were used to
determine the reference values for estradiol, progester-
one, FSH, and LH. Fifteen blood samples were obtained
during a 35-day period at a frequency of three collections
per week. Each volunteer signed a consent form conform-
ing to the hospital ethics committee criteria and com-
pleted a detailed health questionnaire. The women were
not taking anovulant medication and their ages ranged
from 20 to 45 years. All cycles showed a normal profile
ranging from 21 to 31 days.

The longitudinal cycle samples were tested in dupli-
cate by the Immuno 1 estradiol, progesterone, FSH, and
LH assays. The follicular, midcycle, and luteal phases
were established by designating the highest LH value as
day zero. The midcycle phase was represented by the
samples containing the highest LH values. The follicular
phase included the samples after the onset of menses until
the LH surge; the luteal phase followed the LH surge until
the first day of the menses. All cycle samples from each
woman were used for the statistical determination, except
samples that overlap the next cycle. Statistical analyses
were carried out by the parametric method according to
the NCCLS protocol C28-P [18]. The data were previously
transformed to a natural logarithm or square root to
obtain the coefficient of skewness and kurtosis nearest
from zero, determining the best gaussian distribution.

Results

PRECISION STUDIES
The statistical analyses of the precision studies are shown
in Table 1. The within-run CVs ranged from 1.9% to 6.4%
and the between-day CVs from 1.7% to 5.0% for estradiol
concentrations from 116.8 to 6645.8 pmol/L. At the lowest
evaluated concentration of 54.5 pmol/L, within-run and
between-day CVs of 17.9% and 13.8% were observed. This
concentration is below the first calibrator containing es-
tradiol (calibrator 2 with 116.8 pmol/L), and thus was not
used further in evaluation.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Control</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
<th>SD</th>
<th>CV, %</th>
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<td>7.5</td>
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<td>10.6</td>
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<td>36.8</td>
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<td>213.6</td>
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<td>169.1</td>
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<td>Progesterone, nmol/L</td>
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<td>15.6</td>
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<td>TESTpoint 2</td>
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<td>3.4</td>
<td>0.57</td>
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<td>TESTpoint 3</td>
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<td>1.47</td>
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<td>10.1</td>
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<td>10.0</td>
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<td>Pool 2</td>
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<td>Pool 3</td>
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<td>1.7</td>
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<td>0.27</td>
<td>23.5</td>
<td>0.26</td>
<td>22.3</td>
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<td>SETpoint 3</td>
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<td>0.33</td>
<td>5.9</td>
<td>0.43</td>
<td>7.7</td>
</tr>
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<td>SETpoint 4</td>
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<td>0.68</td>
<td>2.2</td>
<td>1.05</td>
<td>3.4</td>
</tr>
<tr>
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<td>SETpoint 5</td>
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<td>1.27</td>
<td>1.9</td>
<td>1.98</td>
<td>3.0</td>
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</table>
The within-run CVs ranged from 1.5% to 10.1% and the between-day CVs from 1.7% to 10.0% for progesterone concentrations from 2.66 to 103.95 nmol/L. At the low progesterone concentration of 1.15 nmol/L, within-run and between-day CVs of 23.5% and 22.3% were obtained, and at 1.28 nmol/L, CVs of 15.2% and 15.6% were observed. This concentration is close to the first calibrator containing progesterone (calibrator 2 with 1.15 nmol/L). The observed CVs are consistent with the manufacturer’s claims.

MINIMUM DETECTABLE CONCENTRATION

The minimum detectable concentration of the Immuno 1 estradiol assay was estimated with 72 reaction rates of the SETpoint estradiol calibrator 1 at 27.1 pmol/L (mean − 2 SDs) and at 35.6 pmol/L (mean − 3 SDs). For the Immuno 1 progesterone assay, the minimum detectable concentration was estimated with 47 reaction rates of the SETpoint progesterone calibrator 1 at 0.51 nmol/L (mean − 2 SDs) and at 0.75 nmol/L (mean − 3 SDs). As noted above, these estimates are based on total imprecision (including both within- and between-run components).

LINEARITY

The results of the dilution linearity studies are shown in Table 2. The tests to verify the linearity quality were made according to the NCCLS protocol EP6-P [19]. Both assays met acceptance criteria proposed by this NCCLS protocol.

METHOD COMPARISON STUDIES

The relation between the Immuno 1 estradiol and DPC estradiol assays is shown in Fig. 1. The regression analysis (Fig. 1A) shows a slope of 1.20, an intercept of −6.4 pmol/L, a correlation coefficient of 0.957, and a $S_{95\%}$ of 147.5 pmol/L. The 95% confidence interval shows that the slope is statistically different from 1.0 (Table 3). Serum samples from patients receiving estrogen replacement therapy gave DPC results higher than those measured by the Immuno 1 assay and are excluded from the linear regression analysis. The cross-reactivity of the antibody used in the DPC estradiol assay with estrone is well-known [20, 21]. Patients receiving estrogen replacement therapy have already been reported to have higher estradiol results by the DPC estradiol assay as compared with other methods [20, 21]. The bias plot representing the relation between the difference and the mean of measurements obtained with both methods is shown in Fig. 1B. The serum samples that showed a higher residual between Immuno 1 and DPC estradiol results have been

<table>
<thead>
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<th>Table 2. Linearity of the Immuno 1 estradiol and progesterone assays.</th>
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<td><strong>Analyte</strong></td>
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<td>Estradiol, pmol/L</td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td>Progesterone, nmol/L</td>
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</tbody>
</table>

* The recovery calculated with the lowest concentration values (SETpoint calibrator 1) is excluded. The two recovered values are the lowest and the highest values obtained for each serum. The high/low numbers are the values obtained for the undiluted serum and the zero calibrators.
tested with the DPC estradiol-6 assay, which offers a lower cross-reactivity with estrone than the DPC estradiol method. The correlation done with 61 samples, including 21 samples from patients receiving estrogen replacement therapy, is presented in Fig. 2. The Immuno 1 results obtained for these samples correlate better with the DPC estradiol-6 results than with DPC estradiol assay. The regression analysis gives a slope of 0.97, an intercept of 110.6 pmol/L, a correlation coefficient of 0.956, and a $S_{y|x}$ of 161.0 pmol/L. The 95% confidence interval of the intercept is statistically different from zero (Table 3).

The correlation between the Immuno 1 progesterone and DPC progesterone assays is shown in Fig. 3. An excellent correlation is observed after excluding 14 serum samples from uremic patients that give discrepant results

| Analyte   | Comparison method | Conc. range | n  | Slope (95% confidence interval) | Intercept (95% confidence interval) | r     | $S_{y|x}$ |
|-----------|-------------------|-------------|----|--------------------------------|-------------------------------------|-------|----------|
| Estradiol, pmol/L | DPC          | 0–2500      | 150| 1.20 (1.14–1.26)                | −6.4 (−45.3–32.6)                    | 0.957 | 147.5    |
| Estradiol, pmol/L | DPC estradiol-6 | 0–2500      | 61 | 0.97 (0.89–1.05)                | 110.6 (48.8–172.3)                   | 0.956 | 161.0    |
| Progesterone, nmol/L | DPC (old)   | 0–130       | 171| 1.23 (1.19–1.26)                | 1.00 (−0.55–2.56)                    | 0.982 | 6.74     |
| Progesterone, nmol/L | DPC (new)   | 0–130       | 45 | 1.28 (1.21–1.35)                | 1.21 (−1.07–3.49)                    | 0.985 | 4.77     |

Fig. 2. Comparison between Immuno 1 estradiol and DPC estradiol-6 assays.

○ serum samples from patients taking estrogen therapy (n = 21). These results are included in the analyses: (A) linear regression and (B) bias plot.

Fig. 3. Comparison between Immuno 1 progesterone and old DPC progesterone assays.

Inset shows the correlation at low progesterone concentrations. ○ serum samples from uremic patients (n = 14). These results are excluded from the analyses: (A) linear regression and (B) bias plot.
between the methods. These results are excluded from the linear regression analysis. The Immuno 1 assay gave slightly higher results than those measured by DPC assay, with a regression slope of 1.23 and an intercept of 1.00 nmol/L (Fig. 3A). A correlation coefficient of 0.982 and a $S_{yx}$ of 6.74 nmol/L were observed.

The Immuno 1 progesterone assay was also compared with the new DPC progesterone method (Fig. 4). The regression line obtained shows a slope of 1.28 and an intercept of 1.21 nmol/L (Fig. 4A). The results from uremic patients were also excluded from the regression analysis. The correlation coefficient ($r = 0.985$) and the $S_{yx}$ (4.77 nmol/L) were similar to those observed with the old DPC progesterone assay. Both correlation studies gave slopes that are different from 1.0 according to the 95% confidence intervals (Table 3).

**REFERENCE VALUES**

The individual hormone profiles from the 29 healthy women showed a typical menstrual cycle pattern. Two representative normal menstrual cycle hormone profiles are presented in Figs. 5 and 6. The reference values estimated by the parametric method are shown in Table 4.

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**Table 4. Reference values for Immuno 1 FSH, LH, estradiol, and progesterone assays.**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Estradiol, pmol/L</th>
<th>Progesterone, nmol/L</th>
<th>FSH, IU/L</th>
<th>LH, IU/L</th>
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</thead>
<tbody>
<tr>
<td>Follicular</td>
<td>72–479</td>
<td>0.4–6.1</td>
<td>3.6–12.1</td>
<td>2.2–12.3</td>
</tr>
<tr>
<td>Mid-cycle</td>
<td>246–1638</td>
<td>0.9–10.7</td>
<td>2.3–20.4</td>
<td>3.5–108.4</td>
</tr>
<tr>
<td>Luteal</td>
<td>135–781</td>
<td>4.8–83.8</td>
<td>1.6–9.8</td>
<td>1.1–19.1</td>
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</table>

$n^*$ represents the number of values used to calculated the reference intervals.
****Discussion****

The within-run and between-day CVs were excellent for both estradiol and progesterone assays, except for concentrations <.66 nmol/L for progesterone and <5.45 pmol/L for estradiol, which gave CVs >10%. The precision performance of the Immuno 1 estradiol assay is superior to the semi- or fully automated assays reported [22–26]. The performance of the Immuno 1 progesterone assay is also better than other reported automated progesterone assays [27–31].

Among the important criteria that influence the desirable analytical performance is the biological variation of a given analyte [32–34]. The recommendation is that an analytical goal for imprecision should be a CV equal to or less than half the within-subject variation for individual single and multipoint measurements. It is interesting to examine the reported studies of biological variation for estradiol and progesterone, even though such studies do not include the possible biological variation for each different clinical circumstance in which the assay might be used. The intraindividual CV for estradiol has been shown to vary from 21.7% to 61.4%, suggesting an analytical goal for CV of 10.9% [34, 35]. All estradiol concentrations evaluated except the lower concentration (54.5 pmol/L) gave CVs within this value. The analytical requirements proposed at the Second Estradiol International Workshop are that the CV be <25% for a working range 150–1000 pmol/L and <10% for a range of 1000–10 000 pmol/L [36]. The intraindividual CV for progesterone has been reported as 13.09% [37], suggesting an analytical goal of 6.5%. The within-run and between-day CVs are lower than this value for progesterone concentrations >5.5 nmol/L.

The minimum detectable concentration for estradiol is equivalent to 24, 26, 38, or better 25, 26, 38, than other commercial automated estradiol assays. For the progesterone assay, the minimum detectable concentration is also equivalent to other commercial automated progesterone assays 27, 28, 30, 31.

The dilution studies performed for the Immuno 1 estradiol or progesterone assays with calibrator 1 are compatible with acceptance criteria proposed by the NCCLS protocol EP6-P [19]. The percentages of recovery and the linear regression analysis demonstrate the excellent linearity of the assays throughout the entire analytical range. No matrix effect was apparent with the manufacturer’s recommended diluent.

The comparison between the Immuno 1 and DPC estradiol assays shows a good correlation between the two methods, although the results obtained from the Immuno 1 assay are slightly higher than those measured by the DPC assay (regression analysis slope statistically different from 1.0). Samples from patients taking estrogens gave DPC results higher than those obtained from the Immuno 1 assay. Estrone has been shown to interfere with the DPC method 20, 21, 39. Because estradiol undergoes first-pass hepatic metabolism to estrone and its conjugated metabolites in patients receiving replacement therapy, the increased DPC values represent cross-reactivity with nonphysiological concentrations of estrone and these metabolites. The results obtained from the Immuno 1 assay correlate better with the DPC estradiol-6 results. This assay is commercialized as having a low cross-reactivity with estrone [40]. These results confirm that the Immuno 1 assay has a low cross-reactivity with estrone.

The results obtained from the Immuno 1 progesterone assay correlate well with those measured by both old and new DPC assays, although the results obtained from the Immuno 1 assay are higher than those obtained by the DPC methods (regression analysis slopes statistically different from 1.0). The serum samples from uremic patients give higher values with the Immuno 1 progesterone assay. Interference by these serum samples with the Immuno 1 assay is suggested by finding progesterone values in males more than three times the upper limit of the reference interval.

Many other categories of serum samples were tested in these correlation studies. For both analytes, no significant difference between the Immuno 1 assays and comparative assays was observed in patient serum samples with increased bilirubin, rheumatoid factor, immunoglobulins or triglycerides, presence of hemolysis, serum samples from postmenopausal women without hormone replacement therapy, healthy men, diabetic patients, pregnant women, or patients receiving stimulation treatment for infertility. In addition, no significant difference was observed with serum samples from uremic patients between Immuno 1 and DPC estradiol methods, and with serum samples from postmenopausal women with hormone replacement therapy between Immuno 1 and DPC progesterone methods.

The regression analysis of correlation studies gave slopes significantly different from 1.0, except for the correlation performed with the DPC estradiol-6 assay, suggesting differences in assay calibration. Indeed, large differences between commercial methods used in routine laboratories for the measurements of these analytes have been reported 38, 41, 42. Consequently, great variability currently exists among laboratories 43. There is a need for a better calibration of these hormone assays between different commercial methods for the same analyte. In addition, the establishment of reference values specific for each method is important for an optimal result interpretation, especially in view of the differences that can exist between commercially available assays. The reference ranges determined for the Immuno 1 assays by the statistical parametric analysis are consistent with literature values 39, 41, 45.

The fully automated Immuno 1 assays for progesterone and estradiol in serum have the technical requirements for their utilization in the routine laboratory. These assays are rapid, with the first result obtained in 38 min, and 120 additional results able to be processed in the following
hour. In addition to allowing the use of nonradioactive reagents, the calibration curve stability of the Immuno 1 assay is at least 2 months, in contrast to the RIA, which requires a new calibration curve in each protocol. Both Immuno 1 assays offer an extended range, allowing minimal number of sample dilutions.

In conclusion, this evaluation of the Immuno 1 estradiol and progesterone assays demonstrates several performance characteristics that contribute to increasing the reliability of these assays. Excellent precision and reproducibility, very good linearity, intermethod correlation, and minimal interference are important criteria for their use in the clinical laboratory.

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References


