Total Estriol in Serum and Plasma as Determined by Radioimmunoassay

C. T. Brooks, J. B. Copas, and R. W. A. Oliver

We present and discuss the results of a statistical study of a set of radioimmunoassay data obtained from parallel determinations of the total estriol content of samples of serum and plasma prepared from 98 specimens of whole blood, taken from pregnant women. Estriol concentrations in the serum exceed by an average of 7% the values found for the plasma prepared from the same whole-blood specimen. The clinical significance of this analytical finding is discussed, and we conclude with the recommendation that serum is the fluid of choice for this assay.

Additional Keyphrases: statistical approach to comparing results for assays of serum and plasma • fetal status

Currently, the most widely used biochemical method for monitoring feto-placental function during the second half of human pregnancy is probably the assay of total estriol in maternal serum or plasma. The method used previously for this purpose consisted of assaying the total estrogen content of 24-h maternal urine specimens. The change to the more convenient assay of fluids prepared from whole blood stems from the introduction of sensitive and specific radioimmunoassay (RIA) procedures (1) and of the subsequent design and commercial availability of RIA assay kits (2).

During the course of a long-term study of the relationships between these two biochemical methods of monitoring feto-placental function (3), we became aware of some short papers (4–6) that showed statistically significant differences between the apparent concentrations of certain compounds in serum and plasma obtained from the same whole-blood sample, when assayed by RIA. Indeed, in one of these papers (5), the authors stated, on the basis of analysis of six whole-blood samples, that “estriol data for none of the plasma tested compare with the results for matching serum.” In this preliminary published study, the plasma was prepared by the use of three reagents, namely, ammonium heparin, oxalate–fluoride, and EDTA, and the values for estriol, as assayed with the Amersham/Searle estriol RIA kit, were always lower than those found in the matching serum.

Because of the possible clinical significance of these preliminary findings, we decided to re-investigate this problem, using (a) a much larger number and variety of maternal blood specimens; (b) heparin only as the anticoagulant for the preparation of plasma, to obviate any adverse effects on the enzyme used in the pre-RIA step in which estriol conjugates are hydrolyzed; and (c) a rigorous statistical study of the analytical findings.

Materials and Methods

The radioassay kits we used were the total estriol RIA kits (IM82) from The Radiochemical Centre, Amersham, U.K. In this kit, estriol conjugates in the blood samples are hydrolyzed by an enzyme preparation, and an 125I-labeled estriol derivative is used concurrently in a sample estimation of estriol by RIA.

An automatic gamma counter was used to count the radioactivity in the radiiodine-labeled samples.

Serum/Plasma Correlation Experiments

Whole-blood samples (10 mL) were taken from 98 pregnant women. Each sample was promptly divided into two 5-mL portions, one of which was placed in a plain glass test tube, the other in a tube containing lithium heparin (product no. LH10; LabCo. Ltd., Marlowe, U.K.). The specimen in the plain glass tube was allowed sufficient time (about 30 min) for clot retraction to occur at room temperature, and then centrifuged (3000 rpm, 10 min); the resulting supernatant serum was transferred with a disposable glass Pasteur pipette to a 5-mL polystyrene test tube. The specimen in the lithium heparin tube was sealed and thoroughly mixed with the anticoagulant by careful manual inversion. The plasma was then obtained by similarly centrifuging this sample tube, and the supernatant plasma was transferred to a 5-mL plastic test tube. The maximum interval between collection of the whole-blood specimen and the production of serum and plasma ready for analysis was 2 h. We then mixed 50-μL aliquots of the serum (or plasma) samples by vortex-mixing for 5 s with 200 μL of the hydrolyzing enzyme solution contained in the Amersham kit, and the resulting mixture was incubated for 2 h at 37 °C. During this interval the mouths of the plastic tubes were covered with Parafilm. After this incubation the tubes were removed from the water bath and the contents were again vortex-mixed to ensure sample homogeneity.

Duplicate 50-μL aliquots of the hydrolyzed sample (and of the hydrolyzed re-constituted serum standards supplied by Amersham) were then pipetted into the bottom of numbered polystyrene tubes, and 200 μL of 125I-labeled estriol solution and 200 μL of anti-estriol serum, contained in the kit, were subsequently added. This mixture was vortex-mixed for 1 s and allowed to stand at room temperature (20 ± 2 °C) for 1 h. We added 500 μL of a 3.16 mol/L ammonium sulfate solution to each tube and thoroughly vortex-mixed. The resulting homogeneous mixtures were then centrifuged at 3000 rpm for 20 min at room temperature and the supernatant fluid was carefully poured off and discarded. The tubes were allowed to drain for 5 min by inverting them on filter paper. The radioactivity in the tubes was then counted in a Wallac gamma counter. We counted the standards used to plot the calibration curve of count vs concentration before and after the unknowns, to check the reproducibility of the counting procedure.

Serum/Serum Correlation Experiments

The above-described analytical methodology was used to assay the total estriol content in duplicate 50-μL aliquots of 42 samples of re-constituted lyophilized serum standards provided by Amersham, to provide a parallel study to the previous serum/plasma correlation study.

Results and Discussion

Assay Statistics

Two pools of pregnancy sera (A and B) were prepared, ali-
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such diagram puter.

quoted in 500-μL quantities, and stored at −20 °C. For pool A the mean total estriol concentration was 141 nmol/L; that of Pool B was 463 nmol/L. We assayed these pooled sera a total of 20 times. Between-batch statistics for this assay are:

<table>
<thead>
<tr>
<th>Pooled sera</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>120–170</td>
<td>141</td>
<td>13.9</td>
<td>9.9</td>
</tr>
<tr>
<td>B</td>
<td>430–530</td>
<td>461</td>
<td>27.6</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Statistical Analysis

For statistical analysis of the assay data we used a computer.

We obtained 98 pairs of measurements of estriol in plasma (x) and in serum (y) (Figure 1). Although the points in this diagram show a clear linear trend, the relationship between x and y is evidently not an exact one and so can only by assessed in terms of statistical methods. Four statistical methods were considered.

Method 1. The conventional approach to analysis of data such as those in Figure 1 is to fit a straight line by least squares. The least-squares line for y as a function of x, for example, gives the best prediction of the value of y in terms of x. In predicting y from x, the value of x is considered to be given in advance and so only y is considered to be influenced by random error.

Conversely, for predicting x from y, only x is considered to be subject to random error, and the least-squares equation is then appropriate with the roles of x and y interchanged. But for describing the relationship between x and y, the subject of this paper, experimental errors in both x and y must be allowed for simultaneously, and straightforward least-squares treatment is inappropriate. Further, the least-squares line is indeterminate, because the choice of the plasma value as x and the serum value as y is arbitrary—had they been labeled the other way round, a different line would have been obtained.

Method 2. If the standard deviation of the experimental error is the same for both the plasma and the serum determinations, and is constant throughout all the data, then the correct modification to least squares is to find the line in Figure 1 that minimizes the sum of squares of the perpendicular distances from that line to each of the points. Equivalently, each value of x (and of y) is assumed to correspond to an unknown “true” value, these “true” values following an exact linear relationship. Differences between observed values and “true” values are assumed to be accounted for by experimental error only. The equations for calculating the estimates of the underlying relationships are given in the Appendix.

Method 3. Inspection of Figure 1 suggests that the experimental errors are not constant but tend to increase over the observational range. In work closely related to this study, Cekan (7) maintained that the experimental standard deviation increases in proportion to the size of the value being measured. In other words, the coefficient of variation (CV) is constant. To allow for this situation, Method 2 above is easily modified by differentially weighting the measurements: the smaller values of x and y are subject to less experimental error and so are given greater weight in the statistical analysis than are the larger values (see the Appendix).

Method 4. A situation intermediate between Methods 2 and 3 is to allow for experimental error to increase with size but at a slower rate than that implied by a constant CV. The fact that RIA procedures give results in terms of counts suggests that a Poisson distribution might be appropriate, in which case the standard deviation of the count would be proportional to the square root of the size of that count. A slight modification to the weights used in the analysis in Method 3 is then needed (see Appendix).

Each of the statistical models in Methods 2–4 was fitted in two different ways, depending on whether the intercept is constrained to be zero. A zero intercept—that is, a direct proportionality y = mx—makes obvious scientific sense, but the

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Table 1. Summary of Results of Various Statistical Treatments of the Analytical Data for the 98 Matching Pairs of Serum and Plasma

<table>
<thead>
<tr>
<th>Model no., and comment</th>
<th>Gradient (m)</th>
<th>Intercept (c)</th>
<th>Correlation coefficient (r)</th>
<th>t-test for unit gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normal least squares of</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) serum vs plasma</td>
<td>1.013</td>
<td>32.2</td>
<td>0.956</td>
<td>0.67</td>
</tr>
<tr>
<td>(b) plasma vs serum</td>
<td>1.109</td>
<td>-18.7</td>
<td>0.956</td>
<td>3.87</td>
</tr>
<tr>
<td>2. (a) Constant error in all measurements</td>
<td>1.063</td>
<td>5.8</td>
<td>0.956</td>
<td>1.95</td>
</tr>
<tr>
<td>(b) As above, but constrained to pass through origin</td>
<td>1.072</td>
<td>0</td>
<td>—</td>
<td>4.74</td>
</tr>
<tr>
<td>3. * (a) Experimentally assessed nonconstant error but constant CV</td>
<td>1.097</td>
<td>-5.4</td>
<td>0.977</td>
<td>3.71</td>
</tr>
<tr>
<td>(b) As above, but constrained to pass through origin</td>
<td>1.070</td>
<td>0</td>
<td>—</td>
<td>4.30</td>
</tr>
<tr>
<td>4. (a) Experimentally assessed nonconstant error intermediate model</td>
<td>1.083</td>
<td>-4.4</td>
<td>0.973</td>
<td>3.27</td>
</tr>
<tr>
<td>(b) As above, but constrained to pass through origin</td>
<td>1.074</td>
<td>0</td>
<td>—</td>
<td>4.80</td>
</tr>
</tbody>
</table>

* CV implied by Model 3(a) and Model 3(b) is 10.8%.
Table 2. Summary of the Results of Various Statistical Treatments of the Analytical Data for 42 Duplicate Pairs of Serum

<table>
<thead>
<tr>
<th>Model no. and comment</th>
<th>Gradient ( (m) )</th>
<th>Intercept ( (c) )</th>
<th>Correlation coefficient ( (r) )</th>
<th>( t )-test for unit gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Serum (I) vs serum (II)</td>
<td>0.995</td>
<td>0.9</td>
<td>0.998</td>
<td>-0.55</td>
</tr>
<tr>
<td>Serum (II) vs serum (I)</td>
<td>0.998</td>
<td>-0.5</td>
<td>0.998</td>
<td>-0.20</td>
</tr>
<tr>
<td>2. (a) Constant error in all measurements</td>
<td>0.997</td>
<td>0.2</td>
<td>0.998</td>
<td>-0.38</td>
</tr>
<tr>
<td>(b) As above, but constrained to pass through origin</td>
<td>0.997</td>
<td>0</td>
<td>-</td>
<td>-0.50</td>
</tr>
<tr>
<td>3.4 (a) Experimentally assessed nonconstant error but constant CV</td>
<td>1.012</td>
<td>-1.4</td>
<td>0.993</td>
<td>0.61</td>
</tr>
<tr>
<td>(b) As above but constrained to pass through origin</td>
<td>0.980</td>
<td>0</td>
<td>-</td>
<td>-1.11</td>
</tr>
<tr>
<td>4. (a) Experimentally assessed nonconstant error</td>
<td>0.999</td>
<td>-0.9</td>
<td>0.999</td>
<td>-0.14</td>
</tr>
<tr>
<td>intermediate model</td>
<td>(b) As above but constrained to pass through origin</td>
<td>0.997</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

*CV implied by Model 3(a) is 7.5%; CV implied by Model 3(b) is 8.3%*

unconstrained line \( y = mx + c \) allows for the possibility of a systematic bias in one or both of the methods.

Table 1 gives the results of fitting the statistical models. The gradients of the two least-squares lines in Method 1 differ substantially, as do the intercept terms, which also differ in sign. The lines fitted by Methods 2-4 are fairly consistent, none of the intercept terms being significantly different from zero. Thus the line can be assumed to pass through the origin, there being no evidence of any systematic bias in the readings. The slopes of the lines for the three constrained models (with \( c = 0 \)) are virtually the same, varying only from 1.070 to 1.074. Thus the correlation coefficient given in Table 1 corresponds to the usual product-moment formula for 1 and 2, but is correspondingly weighted in cases 3 and 4 (see Appendix). The highest correlation, 0.977, occurs for the model in which a constant CV is assumed, which, together with the evidence of Cekan (7) already cited, suggests that Model 3 with \( c = 0 \)—i.e., \( y = 1.07x \)—is the most appropriate. The final column of Table 1 \( (t\)-test) shows that the excess in the slope over unity is statistically significant—hence the conclusion that there is a systematic tendency for values for serum to exceed those for plasma by about 7%, a figure that, although statistically significant, is not large when compared with the size of experimental error. The CV implied by the fit of Model 3 in Figure 1 is 10.8%, a figure not substantially greater than one would expect from repeated assays of the same type.

To check the scientific and statistical methodology used in this study, we also obtained independent data for a series of 42 pairs of measurements in which both values \((x \text { and } y)\) were obtained in serum. Clearly, if our methods are sound, we should obtain the line \( y = x \), i.e., \( c = 0 \) and \( m = 1 \). Table 2 gives the results calculated in exactly the same way as those in Table 1. Confirmation that \( c = 0 \) and \( m = 1 \) is clearly evident, the intercepts all being extremely small and the differences between the values of \( m \) and 1 being well within the limits of statistical error. Further, the CV implied by Model 3 with \( c = 0 \) in 8.3%, not significantly different from the figure of 10.8% found earlier.

We conclude that this combined experimental and statistical study has confirmed the previous report (5) that values for total estriol in serum are, on average, greater than those found in the matching plasma when these are determined by RIA. We believe that these findings are a direct result of the differences between these two fluids rather than being ascribable to the RIA procedures.

We found the actual numerical magnitude of the average difference to be about 7%, nearly twice the value of 4% recently published (without evidence or comment) by the manufac-
Appendix

We give below the formulas for calculating the slope (m) and intercept (c) of the line

\[ y = mx + c \]

for Methods 2-4.

We have \( n \) pairs of readings \( x_i, y_i, i = 1, \ldots, n \). Then

\[ m = \frac{S_{xy}^2 - S_{x}^2 + \sqrt{(S_{xy}^2 - S_{x}^2)^2 + 4S_{x}^2S_{y}^2}}{2S_{xy}} \]

and

\[ c = \frac{\sum w_i y_i - m \sum w_i x_i}{\sum w_i} \]

where

\[ S_{xy}^2 = \sum w_i x_i y_i - \frac{(\sum w_i x_i)^2}{\sum w_i} \]

\[ S_{x}^2 = \sum w_i y_i^2 - \frac{(\sum w_i y_i)^2}{\sum w_i} \]

\[ S_{y}^2 = \sum w_i x_i y_i - \frac{(\sum w_i x_i)(\sum w_i y_i)}{\sum w_i} \]

In these formulae, \( w_i \) is the statistical weight to be attached to the \( i \)th pair of readings, and is given by

\[ w_i = \begin{cases} 
1 & \text{for Method 2} \\
(x_i + y_i)^{-1} & \text{for Method 3} \\
(x_i + y_i)^{-1/2} & \text{for Method 4} 
\end{cases} \]

The weighted correlation coefficient is defined by

\[ r = \frac{S_{xy}}{S_{x}S_{y}} \]

When the line is constrained to pass through the origin—i.e., \( y = mx \)—then the formula for \( m \) is exactly the same as above, except that \( S_{xy}^2, S_{x}^2, \text{and } S_{y}^2 \), are replaced by \( \Sigma w_i x_i^2 \), \( \Sigma w_i y_i^2 \), and \( \Sigma w_i x_i y_i \), respectively.

Rapid Quantitation of Serum Immunoglobulins with a Miniature Centrifugal Analyzer

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For these quantitative turbidimetric assays for immunoglobulins IgG, IgA, and IgM in serum, the reagents used are commercially available in kit form. The two-point assays are performed with a miniature centrifugal analyzer (GEMENI, Electro-Nucleonics, Inc.). The reading taken 5 s after mixing is used to correct for specimen turbidity. Run-to-run precision (CV) was typically 5% (range: 3.0–8.7%). Excellent correlation with radial immunodiffusion was observed for each test \((r > 0.98 \text{ for each test})\). The dynamic range of each test extends above and below the reference-interval ranges.

Additional Keyphrases: “kit” methods · reference interval · turbidimetry · variation, source of · effects of changes in antiserum

Several authors describe use of the centrifugal analyzer to quantitate IgG, IgA, and IgM in serum \((1–5)\). These methods are all based on the apparent increase in absorbance (light scatter) during the immunoprecipitin reaction. Past authors prepared their own reagents (specific antiseria and polymeric enhancers) from commercially available source materials \((1)\). Variations in specificity and titer found in commercially available antiseria necessitate changes in protocol for each new batch of antiseria. We have investigated, in a routine laboratory setting, the performance characteristics and use of a recently developed set of optimized reagents and standards for the turbidimetric quantitation of immunoglobulins in serum \((Electro-Nucleonics, Inc., Fairfield, NJ 07006)\). The reagents used are adjusted for titer variation so that the lyophilized antiserum in each vial is optimal. Thus, standard curve response is consistent from lot to lot of reagent.

**Materials and Methods**

We used the GEMENI miniature centrifugal analyzer \((Electro-Nucleonics, Inc.)\) and GEMENI Immunoglobulin Assay kits for IgA, IgG, and IgM. Each kit contains five vials of lyophilized goat anti-human, heavy chain, monospecific antiserum and one vial of accelerator reagent in powder form. The accelerator reagent, polyethylene glycol, is reconstituted in distilled water and this solution is used to dissolve the lyophilized antiserum. The final concentration of the accelerator is 20 g/L for the IgG and IgM assays, and 30 g/L for IgA.

Antiseria produced against purified human immunoglobulins \((IgG, IgA, \text{and } IgM)\) were rendered monospecific by absorption with heterologous immunoglobulins. Antiserum to IgG was absorbed by multiple passage over a column of Fab\(_2\) \((6)\) coupled (by the cyanogen bromide method) to Biogel A 0.5m \((Bio-Rad Laboratories, Richmond, CA 94804)\) \((7)\). Antiseria to IgA and IgM were absorbed by multiple passage over an affinity column of IgG coupled to Biogel \((8)\). Some antiseria to IgM required further absorption with a column containing bound IgM from cord serum \((9)\).

Each run was standardized with five GEMENI Immunoglobulin Standards covering the following ranges \((mg/L)\): IgG 1500–25000, IgA 250–4500, and IgM 350–4500.

We compared the results with results obtained with Kal- lestad radial immunodiffusion "Endoplates," either regular

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