Urinary Estrogen Determination in Pregnancy

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Results of an extensive trial of a quick fluorometric determination of estriol in pregnancy urine are described. The method is compared with the much more laborious one of Oakey et al. [Clin. Chim. Acta 15, 35 (1967)]. Trial of another apparently simpler method indicated it to be unsatisfactory in practice. The method described is accurate, simple, and rapid.

Additional Keyphrases intercomparison of methods • fluorometry • interference by glucose • fetal malfunction

The use of urinary estrogen assay in pregnancy as a measure of fetal well-being is now widespread. Estriol is considered by many workers to be the compound of greatest importance, since both fetus and placenta appear to share in its production (1). Pregnanediol excretion, used as an index by some workers, is reputed to be produced by the placenta alone and can be normal when fetal malfunction would be indicated by the estriol values (2). However, specific methods for determining estriol, such as that of Brown (3), are too long and complex for routine use in the average hospital laboratory, which is faced with daily determinations on large numbers of specimens from a maternity unit.

Some simplification has been introduced previously by estimating total estrogens, of which estriol constitutes some 80% during late pregnancy. The basic work on which most of these simplified methods is based is that of Ittrich (4), who described a shortened purification procedure followed by measurement of the estrogens by the Kober reaction (5). These methods were studied by Oakey et al. (6), who produced a method for estimating total estrogens to monitor fetal risk in pregnancy. Although an improvement on previous methods, this is still fairly laborious and involves a number of steps in which less than skilled technique can result in loss of estrogens, although internal standards were introduced to allow for this. Using this method, one skilled technician can estimate at most 15 specimens per day, including duplicates and standards. An internal standard is necessary, not only because of manipulative losses but also because some substances—such as glucose—frequently present in pregnancy urine, interfere with the production of the Kober chromogen, and hence the results would be erroneous.

Gel filtration removes such interfering substances in the method of Eechante and Demeester (7), but this again adds to the length and complexity of the analysis.

Methods in which gas chromatography is used for the final determination have good sensitivity, but require extensive purification procedures.

A combination of simplicity, speed, and adequate sensitivity and accuracy was claimed for the methods of Skramovsky and Haeberle (8) and of Brombacher et al. (9). We investigated these methods. The method of Brombacher et al. (9) proved to be superior, and was compared extensively against the method of Oakey et al. (6), some simplification being introduced. Precision and freedom from interference were investigated.

Materials and Methods

Reagents

Stock estriol standard. Dissolve 1 mg/ml in Spectrosol grade ethanol.

Working estriol standard, 10 mg/liter. Dilute 1 ml of stock solution to 100 ml with doubly deionized water. Prepare freshly at least once a week.

Hydroquinone. Dissolve 2 g of AR hydroquinone in 200 ml of AR concentrated sulfuric acid. Com-

Complete solution requires 3 to 4 h at 4°C. Store in a dark bottle at 4°C. Do not prepare larger quantities, as there is a tendency for brown discoloration to develop on storage, owing to exposure to light.

**p-Nitrophenol reagent.** Dissolve 10 g of p-nitrophenol in 500 ml of an-grade chloroform. Store in a dark bottle at 4°C. If many determinations are being run it is convenient to dissolve 50 g of p-nitrophenol in a 2.5-liter Winchester bottle of an-grade chloroform. A brown glass automatic dispenser (500-ml reservoir, with 10-ml syringe, set at 6 ml) is used solely to dispense this reagent.

**Doubly de-ionized water.** This is used for all operations.

**Apparatus**

**Fifteen-milliliter glass-stoppered test tubes.** These are stored in 10-fold diluted sulfuric acid in doubly de-ionized water, and used for this purpose only. Immediately before use they are removed from the acid, washed well in running tap water followed by six rinses in doubly de-ionized water. They are then dried at 95°C in a hot-air oven.

**Semiautomatic (construction) pipet, 10 µl.**

**Automatic dispenser.** Reservoir, 250 ml; syringe volume, 2 ml, set at 1 ml, is retained to dispense doubly de-ionized water.

**Automatic dispenser.** Reservoir, 250 ml; syringe volume, 5 ml, set at 3.75 ml. Retained to dispense doubly de-ionized water.

**Clinical laboratory centrifuge.** To accommodate 15-ml tubes.

**Acetone.**

**EIL fluorometer, model 27 C, or equivalent.** The dynode voltage is adjusted to give satisfactory reading of standard at intermediate sensitivity. In our case, a reading of 50 dynode volts gives sample sensitivity on coarse sensitivity range 3. Higher dynode voltages tend to give considerable instability of the pointer. The lamp is set at 60 V. For excitation, the interference filter is 538 nm, the emission filter an Ilford 202.

**Method**

Each item is run in duplicate (with use of doubly de-ionized water).

**Blank.** One milliliter of water.

**80 mg/liter standard.** 0.02 ml of working standard + 1.0 ml of water.

**10 mg/liter standard.** 0.01 ml of working standard and 1.0 ml of water.

**Urine.** 0.01 ml of urine and 1.0 ml of water.

The same automatic pipet is used to measure all the small volumes. The 1.0 ml of water is dispensed into each tube from the automatic pipet, 2.0 ml of hydroquinone reagent is added and the contents of the tubes are mixed, without stopping. The tubes are then placed in a vigorously boiling-water bath for 45 min. Immediately after placing the tubes in the bath, switch on the fluorometer, which allows about 1 h for it to stabilize. At the end of 45 min, remove the tubes from the water bath and immediately place them in a bath of ice water. After about 30 s add 3.75 ml of water from the automatic dispenser to each tube in turn, mixing well but carefully to ensure mixing of the acid and water. Each tube is returned to the ice water immediately after mixing. When water has been added to all the tubes, the first tubes will be cool and ready for the careful addition of 6 ml of the p-nitrophenol–chloroform reagent from the automatic dispenser.

From this point to the final reading, all operations should be carried out without delay. The p-nitrophenol–chloroform reagent can be added to single tubes or to as many as four tubes at a time, depending on the facility of the technician. The tubes are stoppered and immediately shaken by hand vigorously for 20 s. They are then returned to the icewater bath. The next few tubes then have the p-nitrophenol–chloroform reagent added. If the number of tubes in the batch exceeds the capacity of the centrifuge, those tubes which will just fill the centrifuge are placed in it as soon as p-nitrophenol–chloroform reagent has been added to this number of tubes. After removing the stoppers, the centrifuge is switched on, allowed to reach a speed of 3000 rpm, then switched off and allowed to stop. During this time p-nitrophenol–chloroform reagent can be added to the remaining tubes that are awaiting similar centrifugation.

After centrifugation, the upper (aqueous) layer is aspirated and discarded. Since the 6 ml of chloroform solution exceeds that needed for fluorometry, as much as 1 ml of the chloroform layer may be sacrificed to ensure that as much as possible of the aqueous phase is removed. The tubes are maintained at room temperature until all the tubes are processed in this way, at which time all tubes are ready for fluorometry. The fluorometer cells which have been stored in water are thoroughly rinsed in water, then dried by several successive washes in acetone and standing in air.

The fluorometer—with the correct filters in place—is zeroed.

Test solutions are more carefully added to fluorometer cells by gently pouring the chloroform solution down the inside of the cuvet, with great care to avoid introduction of bubbles of aqueous phase. Any chloroform solution spilled on the outside of the cuvet must be removed immediately before reading, by partly immersing the cuvet in water and drying it with a soft cloth. After the same cuvet has been used for about 10 different test solutions, any trace of water should be removed from it by thorough rinsing with acetone.
The first solution to be introduced into the fluorometer is the blank, on which the fluorometer is zeroed when set at maximum sensitivity.

The 20 mg/liter standard solution is then introduced and the fluorometer sensitivity decreased until this solution reads 20 (on a scale which extends to 100). The 10 mg/liter standard solution is introduced and read, with the same sensitivity setting. It should, of course, read 10.

All the duplicate standards are read. If a minor discrepancy does occur with one tube, this is ignored and the instrument set on the three which do agree.

The unknowns are then read serially, the standards and blank being checked half way through the batch and again at the end.

Notes

The standard curve is linear to at least 100 mg/liter. Two points are thus ample for standardization. The two values noted above were adopted as most convenient for 24-h urines that had been prediluted to 2000 ml. However, we prefer to use urines undiluted, noting the 24-h volume. In this case, working standards of 10 mg/liter and 30 mg/liter might well be preferred, as most values would then lie between these standards, although in our experience no difference is noted.

Use of internal standards was found to be unnecessary.

The filters used were not purchased specifically for the purpose; they were the ones at hand in the laboratory that seemed to be most nearly optimal.

A batch of 20 specimens can be analyzed in about 3 h, including the time for washing and drying the tubes. One technician can analyze 60 specimens per day (in duplicate—i.e., 120 singles). A large volume of urine of low estriol content—such as 7 mg/liter—is stored frozen as a series of small aliquots. One of these is thawed and included as a control with each batch.

The normal range used is that given by Oakey et al. (6).

Urine for estrogen analysis should be stored at 4°C, and the analysis should be done promptly.

The method of Skramovsky and Haeberle was used as described in their paper (8).

Results

Tests of the method of Skramovsky and Haeberle (8) showed it to work well on pure estriol standards and to give a satisfactory fluorescence curve, but it was soon apparent that there were some samples for which there was a gross discrepancy between the two methods. Some urines had a fluorescence less than that of the reagent blank, and frequently this was suspected prior to measurement because the solution was charred after the heating with sulfuric acid. The presence of glycosuria seemed a likely cause. This was tested experimentally by adding glucose to an aliquot of urine, then analyzing the urine with and without added glucose. The presence of glucose caused charring and quenching of the fluorescence. Glucose is a very common contaminant of urines in late pregnancy and its removal would add considerably to the time and difficulty of the determination; furthermore, it seemed probable that substances other than glucose had a similar effect. Therefore, the method was abandoned since it was not usable in its simple form. The work of Contractor et al. (10) indicates that, with adequate purification procedures before the heating with acid, the method works satisfactorily.

The method of Brombacher et al. (9) was similarly investigated. Our technique is described at length, as failure to attend to detail can result in errors.

Dr. Oakey was kind enough to let us have a series of urines analyzed in his laboratory by his method.

Figure 1 shows a plot of the results of analysis of 133 urines by the two methods. The 45° line, on which values would lie if the two methods gave identical results, is included, and indicates excellent agreement between the methods at values between 0 and 15 mg/24 h. Above this level of excretion the Brombacher technique appears to give higher values. These results were statistically analyzed by Dr. H. Braunsberg, to whom we are indebted for the following comments:

(a) The correlation coefficient for the two methods was 0.91. (b) The regression line, with the figures from the Oakey method as x and the Brombacher method as y, gives the relation

\[ y = 1.38 + 1.073 \, x \]

with 95% confidence limits for the slope of 1.07 ± 0.08. The intercept value does not differ significantly from zero. The slope
was tested against the hypothesis that $\beta = 1$; $P$ was less than 0.05, indicating that the slope is unlikely to differ from 1. (c) The mean difference was 2.93 mg/24 h (Brombacher > Oakey), and differed significantly from zero ($P < 0.001$). Thus, the Brombacher method does seem to give significantly greater values than the Oakey method.

However, if one breaks down the results into ranges of different levels of excretion the results are as follows.

In Table 1 the one-sample t-test (paired data) was used to calculate mean difference ($\bar{d}$), Students $t$, and the probability ($P$) of finding the particular mean difference by chance if there were no actual difference (null hypothesis). $N$ is the number of pairs of results used in each comparison.

Thus, for excretions up to 15 mg/24 h there is no significant difference between the two methods (Table 1). For greater excretion rates the Brombacher method gives values that are significantly higher than the Oakey method.

Precision

A study was made of the results for some 300 urines, analyzed in duplicate under normal routine laboratory conditions. Figure 2, a plot of these results, illustrates the excellent precision at all levels of estrogen. The values were divided into a series of groups of increasing estrogen level and the value for the standard deviation and coefficient of variation at the mean level for each group was determined (Table 2).

Discrepancies between Brombacher and Oakey Methods

When it became obvious that the results by the Brombacher method were often somewhat higher than those of the Oakey method, possible causes of this were investigated. The most obvious source of erroneously high results would be the presence of some other highly fluorescent substance in the final solution. The method of Brombacher relies on the fact that, at high dilution, compounds other than estriol will be present in amounts too small to interfere. In an attempt to confirm this, we examined the excitation and fluorescence spectra of a series of solutions on an Aminco-Bowman spectrophotofluorometer. The spectra of the Kober chromogens produced from pure estriol solutions were compared with spectra from urines that showed agreement by the two methods and ones showing a large discrepancy.

In every case, identical spectra with single peaks were obtained for excitation and emission. Thus, augmentation of the fluorescence by substances other than estriol could only be postulated if the interfering compounds invariably had both excitation and emission bands occurring at the same wavelength as estriol—a most unlikely assumption. The fluorescence excitation and emission spectra were plotted over the range 200 to 800 nm. At the dilution of urine used in this technique no fluorescence was detectable outside the estrogen peaks. This may be taken to confirm the validity of the assumption made by Brombacher et al. (9) in making such a high dilution of urine.

Discussion

The method has been shown to work well under routine conditions. The results, in particular those below 15 mg/24 h, which is the most important excretion rate from a clinical point of view, are strictly comparable to those obtained by

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**Table 1. Statistical Comparison of Brombacher and Oakey Methods for Urinary Estrogens**

<table>
<thead>
<tr>
<th>Range, mg/24 h</th>
<th>$\bar{d}$</th>
<th>$N$</th>
<th>$t$</th>
<th>$P$</th>
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<tbody>
<tr>
<td>0-15</td>
<td>0.05</td>
<td>33</td>
<td>0.12</td>
<td>not significant</td>
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<tr>
<td>15-30</td>
<td>2.54</td>
<td>44</td>
<td>4.11</td>
<td>$\pm 0.001$</td>
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<td>50+</td>
<td>5.81</td>
<td>25</td>
<td>3.58</td>
<td>$\pm 0.001$</td>
</tr>
<tr>
<td>All</td>
<td>2.93</td>
<td>133</td>
<td>5.86</td>
<td>$\pm 0.001$</td>
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</tbody>
</table>

**Table 2. Results for Duplicate Determinations of Urinary Estrogens**

<table>
<thead>
<tr>
<th>N</th>
<th>Range, mg/24 h</th>
<th>Mean level</th>
<th>SD</th>
<th>CV, %</th>
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<tr>
<td>20</td>
<td>2.5-7.4</td>
<td>5.0</td>
<td>0.46</td>
<td>9.1</td>
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<td>48</td>
<td>7.5-12.4</td>
<td>10.0</td>
<td>0.71</td>
<td>7.1</td>
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<tr>
<td>45</td>
<td>12.5-17.4</td>
<td>15.0</td>
<td>0.83</td>
<td>5.5</td>
</tr>
<tr>
<td>59</td>
<td>17.5-22.4</td>
<td>20.0</td>
<td>1.13</td>
<td>5.6</td>
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<tr>
<td>44</td>
<td>22.5-27.4</td>
<td>25.0</td>
<td>1.07</td>
<td>4.2</td>
</tr>
<tr>
<td>26</td>
<td>27.5-32.4</td>
<td>30.0</td>
<td>1.48</td>
<td>4.9</td>
</tr>
<tr>
<td>19</td>
<td>32.5-37.4</td>
<td>35.0</td>
<td>2.02</td>
<td>5.8</td>
</tr>
<tr>
<td>12</td>
<td>37.5-42.4</td>
<td>40.0</td>
<td>1.68</td>
<td>4.2</td>
</tr>
<tr>
<td>25</td>
<td>42.5-57.4</td>
<td>50.0</td>
<td>1.70</td>
<td>3.4</td>
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the Oakey method. As the latter has been accepted as a satisfactory method for estimating estrogens as a measure of fetal viability, it follows that the present method should also be similarly acceptable. The precision is excellent. Speed and simplicity are important in this context. Using the simplified procedure described here, one technician could easily handle the requests from an average-sized maternity unit.

We suggest that the results by the Brombacher method are more likely to represent the true estrogen content.

The method of Oakey et al. is admittedly subject to losses of steroid by destruction in the heating processes, which exceed those of the Brombacher method by 1 h at 100°C, and also possibly in the succeeding manipulations. To correct for these losses an internal standard is introduced but in fact the standard is not introduced until after the hydrolysis step, and losses up to this point are thus not measured. In the Brombacher method, as carried out by us, the standards receive the same treatment from start to finish, and consequently will assess the method in entirety.

The method is accurate, precise, and sensitive. It is significantly quicker and simpler than the method of Oakey et al. and can be recommended for adoption in clinical laboratories.

Our thanks are due to Dr. Oakey, Division of Steroid Endocrinology, University of Leeds, for providing samples of urine with results of analysis by his technique, and for his helpful discussions. We also thank Dr. H. Braunsberg, M. R. C. Clinical Endocrinology Research Unit, Edinburgh, for some of the statistical calculations.

References


