Urinary Estrogens in Pregnancy

Improved Method for Their Determination in Humans

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One of the difficulties encountered in the determination of urinary estrogens arises from the fact that no procedure so far developed is capable of cleaving the estrogen conjugates quantitatively without destroying any of the free steroids (1). Reporting on a thorough investigation of this subject Van Bruggen (2) stated: “The use of butanol as the solvent and hydrolyzing medium . . . appears to be of definite promise.” Following this suggestion, an improved method has been developed for the determination of estrogens in the urine of pregnant women. This method yields estrogen values 10–30 per cent higher than those obtained by conventional methods.

REAGENTS

The n-butanol to be used must be tested for the presence of impurities which destroy the estrogens during hydrolysis. Reagent-grade butanol cannot be assumed to be free of such impurities. Butanol of satisfactory quality was prepared by refluxing 1 L. of butanol with 1 Gm. of o-phenylene diamine and 50 ml. of concentrated HCl for 24 hours. The butanol was then distilled off in a current of steam.

METHODS

Extraction

An aliquot of the urine specimen, about 500 ml., is acidified to approximately pH 2 with phosphoric acid, saturated with butanol, and placed...
in tube A of the tube-air lift extractor shown in Fig. 1. The butanol (50 ml.) is added at B, the stopper (C) is placed in position, the tube (D) being adjusted to just touch the upper surface of the butanol in A. A slow current of air is introduced through the right-angle tube (E), which escapes via D, carrying with it drops of butanol. The rate of flow of the butanol is regulated by the screw clamp (F) to the maximum attainable without causing an emulsion to form in the upper part of column A. Approximately twenty minutes are required to attain 99 per cent of the equilibrium concentration of methyl red in the butanol phase. In the case of the estrogen conjugates 45 minutes are allowed for equilibration. Finally all butanol is collected in A by removing C, D, and E and applying air pressure at B. The butanol is drawn off as completely as possible by pipet.

The urine is extracted five times, using 50 ml. of n-butanol saturated with water for each extraction. The extracted urine is discarded. The acidity of the aqueous layer is checked after each extraction and adjusted to approximately pH 2 by adding further quantities of phosphoric acid. Although occasional urine specimens form emulsions involving part or all of the butanol layer unless the extractor is run extremely slowly, emulsion formation can be disregarded in adjusting the flow rate, if the emulsion is removed and filtered at the end of the first extraction. The aqueous layer of this filtrate is returned to the extractor, and the butanol layer is combined with the unemulsified butanol, if any. Emulsions
Table 1. Comparison of Results of Aqueous- and Butanolic-Hydrochloric Acid Methods of Hydrolysis in Urinary-Estrogen Determination

<table>
<thead>
<tr>
<th>Urine sample</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous hydrochloric acid</td>
<td>6.65*</td>
<td>21.4</td>
<td>9.7</td>
<td>8.6</td>
<td>9.4</td>
<td>1.05</td>
<td>9.1</td>
<td>7.6</td>
<td>14.0</td>
<td>6.6</td>
</tr>
<tr>
<td>Butanolic hydrochloric acid</td>
<td>7.55</td>
<td>27.9</td>
<td>11.3</td>
<td>10.5</td>
<td>10.4</td>
<td>0.91</td>
<td>9.9</td>
<td>11.4</td>
<td>28.0</td>
<td>12.3</td>
</tr>
</tbody>
</table>

* All values are in milligrams per liter.

will not be formed in subsequent extractions, because this one filtration removes the emulsion-stabilizing solids from the urine.

Separation and Assay

The combined butanol extracts are heated to boiling under reflux. Concentrated HCl is added through the condenser, in an amount to equal 15 per cent of the combined solvent extract, and the refluxing is continued for 1 hour. After cooling, the acidified butanol is brought to approximately pH 7 by the slow and careful addition of concentrated NH₄OH. The butanol is distilled off in a vigorous current of steam, and by supplying additional heat from a burner. The volume of the resulting aqueous solution is arranged to be 50 to 100 per cent of that of the butanol originally present. To the warm aqueous solution, toluene, in amounts equal to one tenth the volume of the aqueous solution, is added, and the contents of the distilling flask are transferred quantitatively to a separatory funnel. Small particles of tarry matter are dissolved in a few milliliters of ethanol. The estrogens are then concentrated and assayed as described previously (3).

RESULTS

The estrogen values obtained were higher than the values obtained by the previous methods (3), with the exception of Sample F, which was from a patient with severe preeclampsia and very low estrogen output (Table 1).

DISCUSSION AND CONCLUSIONS

Venning (4) and Stimmel (5) have shown that the conjugated estrogens in urines of pregnant women can be extracted quantitatively with four portions of one-tenth volume of butanol from urines acidified to Congo red. This corresponds generally with our experience; but a qualification
needs to be made, which applies to all deductions based on experiments with urinary estrogens—as the properties of the conjugated estrogens vary considerably from one urine sample to another, something like a dozen urine specimens from different individuals should be tested, preferably including cases with abnormalities such as toxemia of pregnancy, before a given method can be accepted as consistently reliable. It needs to be specified further that the urines extracted with butanol as described above did not contain any estrogens as determined by the Allen-Kober test (6). We have encountered one urine specimen that retained 4 per cent of its estrogens after four extractions with one-tenth volume of butanol, and we now use five extractions routinely.

Van Bruggen (2) first called attention to the possible advantage of hydrolyzing the conjugated estrogens in a medium of butanol. This has been confirmed. The cleavage in butanol has been found to be complete after 60 minutes' boiling with acid in all urines tested so far, while complete cleavage of the conjugates in the same urines, in an aqueous medium, required boiling for 90 minutes or more. Also, the free steroids are more stable in hot butanolic acid than in aqueous acid, the rate of destruction of estrogens in butanol being about 1 per cent per hour versus 2–6 per cent in aqueous acid.

Extraction with butanol and hydrolysis in this solvent, therefore, seem to be quite efficient operations. The reason that they have not been more widely adopted seems to be the frequent occurrence of very stable emulsions of butanol and urine at pH 2, which have to be broken by centrifugation, a time-consuming step. The need for this has now been eliminated by the use of the tube-air lift extractor.

SUMMARY

A new method has been described for the cleavage of the estrogenic steroid conjugates occurring in the urine of pregnant women. It yields higher estrogen values than the methods which have been employed in the past.

REFERENCES