Measurement of Urinary Estrogens

R. Hobkirk

In a commentary on the existing methodology for the determination of urinary estrogens, data are given for normal ranges in nonpregnancy and pregnancy urine as well as the expected interferences due to medication and substances occurring in high concentrations in disease states. Because of the wide range of normal excretion values, the point is made that only serial analyses of a patient's physiology course (e.g., in pregnancy associated with diabetes) are of clinical value.

Modern estrogen methodology dates back only some 5 years to the time when Brown reported a chemical method for the measurement of the three "classic" urinary estrogens—estriol, estradiol-17β (estradiol), and estrone (1). This was followed closely by the method of Bauld (2) which yields substantially the same results as does Brown's procedure. These were the first technics capable of measuring, with any accuracy, the small amounts of these three compounds occurring in nonpregnancy urine.

Methods of Brown and Bauld

Both of these methods are based on the acid hydrolysis of the conjugated estrogens in 1/5 of a 24-hour urine volume, with extraction of the free estrogens and purification of the extracts, taking advantage of the phenolic properties of the compounds in question. This is followed by simple partition between benzene and water, yielding an estriol-containing aqueous phase and a benzene-soluble estrone-estradiol fraction. At this point the procedures diverge. The Brown method involves methylation of the two fractions in an alkaline medium, yielding the estrogen methyl ethers. This step is of extreme
value in purification since it results in a marked decrease in polarity of the estrogens, permitting removal of polar impurities by simple solvent partition. Moreover, it confers upon the molecule a much increased stability, allowing destruction of further impurities by treatment with hydrogen peroxide, without harm to the estrogens. Additional purification of the methyl ethers of estrone and estradiol, and separation from each other, is achieved by adsorption chromatography on small alumina columns using petroleum ether:benzene solvent systems. Bauld's method, following the benzene:water partition, involves saponification of the estriol fraction with subsequent re-extraction, and considerable purification of the estriol after pH adjustment. Finally, this fraction is chromatographed on a celite partition column in the solvent system ethylene dichloride:methanol:water. The estrone-estradiol fraction is separated into its two components by a similar column partition in the systems benzene:sodium hydroxide and ethylene dichloride:benzene:sodium hydroxide. These chromatographic steps result in the removal of considerable amounts of pigmented impurities both more polar and less polar than the estrogens themselves. The estrone and estradiol fractions are finally saponified and re-extracted in readiness for quantitative measurement.

In both of these methods the final measurement is by a modified Kober color reaction of considerable specificity (3), reading the absorbance at three wave lengths with correction for nonspecific chromogens by the application of Allen's equation (4). In order for this correction to be valid the "background color" (chromogenicity not due to the estrogens) must exhibit a linear absorption spectrum over the range in which the readings are to be made (480-545 mµ). This has been shown to be so for urinary fractions in the absence of interfering drugs (1, 2, 5).

The reliability of these two methods is about the same. Accuracy, as measured by the recovery of added free estrogens after the hydrolysis step, ranges from 75 to 90%. The absolute lower limit of sensitivity is less than 1 µg./24 hr. for each of the three estrogens, but at this level quantitative significance is lacking. At the 3 µg./24 hr. level the maximum error of a measurement carried out in duplicate is ±25% while at the 5 µg./24 hr. level and upwards the possible error is ±15% or less. Both procedures yield similar results when applied to the same nonpregnancy urines (6). Moreover, the method of Brown has been compared with bioassay (7), countercurrent dis-
tribution (8), and isotope dilution technics (8) with good results. The specificity of both methods is adequate in most instances but Brown's procedure is adversely affected by medication in the form of senna, phenolphthalein, cascara, meprobamate, and stilbestrol (9). Under such circumstances high, low, or even negative values may be obtained, particularly for estriol and estradiol. Moreover, high cortisone dosage can cause low values for both estriol and estradiol, and similar results are found in patients whose neutral steroid excretions are very much elevated, as in adrenal carcinoma. Because of this interference a saponification step, modeled on that employed in Bauld's method, has been introduced into Brown's procedure prior to methylation (9). This additional treatment, although improving the situation considerably, is not completely capable of removing contamination in all cases. It is a good rule, therefore, to withhold such medication when estrogen analyses are to be performed. This is also true of Bauld's method although cortisone and stilbestrol have not been shown to interfere with it.

Both methods have, of course, definite advantages and disadvantages associated with them. It may be said from the standpoint of the clinical chemistry laboratory that both are time-consuming and require a high degree of technical skill for successful performance. For example Brown's method requires the use of alumina of a specific moisture content which must be checked regularly if success is to be assured. Likewise, preparation of the partition columns in Bauld's procedure requires considerable uniformity, and thermostatic control is necessary during chromatography. Both methods are advantageous in not requiring really expensive equipment.

Estrogens in Nonpregnancy Urine

Application of the above technics has enabled the attainment of a much more accurate picture of estrogen excretion during the normal menstrual cycle than has hitherto been possible. Two peaks in excretion occur (10), one at approximately 14 days (ovulation peak) the other at about 22 days (luteal peak). At the onset of menstruation the three estrogens together total only some 5-25 μg./24 hr. (11). At the two peaks the total is in the range 25-100 μg./24 hr., the levels during the ovulation peak being usually rather higher than at the luteal peak. Estradiol accounts for only some 10 per cent of the total during the menstrual cycle, whereas the ratio between estriol and estrone is generally slightly in favor of the former. Levels of
these three estrogens in the urine of men are similar to those during the onset of menstruation in the normal female, while in the postmenopausal female rather lower values are encountered (11). For the nonpregnant state the estrogen pattern existing in the urine is considered to represent metabolites of secreted estradiol and/or estrone (12). During the menstrual cycle most of the urinary estrogen is of ovarian origin but a certain fraction is contributed by the adrenal cortex. Bilateral adrenalectomy reduces urinary estrogens to a significant extent (13, 14) while ACTH stimulation of individuals with intact adrenals results in positive response (15, 16).

Brown et al. have reported on the value of the chemical measurement of urinary estrogens in gynecologic disorders (17) but it is necessary to perform many serial analyses before any diagnosis can be established. Because of this and because of the lengthy nature of the chemistry involved, it is likely to be certain modified technics which find application in the clinical chemistry laboratory. Among these may be mentioned a method for the measurement of urinary estrone alone (18), this being modified from the Bauld method. This is a relatively quick procedure. It may be pointed out here that although the separation and measurement of estriol, estradiol, and estrone has assisted considerably in elucidating the problem of estrogen metabolism, no particular clinical advantage has been derived which could not have been obtained by measuring all three together, had this been possible. This is because of the very similar qualitative pattern observed for all three in nonpregnancy urine.

**Estrogens in Pregnancy Urine**

Both of the methods considered above are capable of being modified for pregnancy urine (18, 19). Because of the high estrogen levels occurring under these conditions small urine volumes (10–20 ml.) suffice for analysis, with a subsequent decrease in the volumes of solvents and other reagents required. Moreover, since estrogen excretion increases during pregnancy without an accompanying rise in contaminants, it is possible, for example in Bauld’s method, to simplify the procedure by omitting column chromatography of the estriol and saponification of the other two estrogen fractions (18).

Brown made a study of the excretion of the three “classic” estrogens throughout normal human pregnancy (19) in which he found estriol levels to increase some one thousand-fold compared with the nonpregnant state. The average estriol levels in late pregnancy
amount to 35 mg./24 hr., while estrone and estradiol, although increasing considerably, only reach some 2 mg. and 0.7 mg./24 hr., respectively. The relatively greater increase in estriol is now usually believed to be due to the secretion of this compound as such by the placenta, besides that of estradiol and/or estrone (12).

One area in which urinary estrogen analyses may be of value is in pregnancy associated with diabetes. Because of the high incidence of intrauterine death occurring in this disorder an investigation was made of the levels of estriol, estradiol, and estrone in normal and diabetic pregnancies (20). No marked difference was observed in the estrone and estradiol levels from one group to the other but there was a tendency for estriol to be lower in the diabetic subjects. Also, further work in this laboratory has indicated that in diabetic pregnancies where intra-uterine death occurs there is a tendency for urinary estriol to be rather low (10 mg./24 hr. or less) over the period 28-35 weeks with a failure to show the normal rise during this time. Weekly analyses of estriol levels over this period may be a useful guide as to the desirability of performing sections on such individuals. Sharp falls in estriol excretion during pregnancy are usually associated with prior fetal death and are of little or no use clinically. It may be emphasized that a single 24-hour measurement of estrogens in pregnancy is of virtually no value in a clinical sense. Serial analyses are required because of the wide spread in normal values and the variation in day-to-day excretion.

Urinary estriol in pregnancy has previously been suggested as a good index of placental function (21). The chemical procedure is not difficult and may be performed within a working day. An alternative method for estriol measurement has been described by Eberlein et al. (22). This was devised as a means of estimating estriol in non-pregnancy but could conceivably be applied during pregnancy. It involves enzyme hydrolysis, suitable extraction procedures, saponification, alumina column chromatography, and fluorimetric analysis.

Hydrolysis of Estrogen Conjugates

An important step in the analysis of steroids in general is the hydrolysis of conjugates (glucuronides and sulphates) prior to extraction. The classic means of hydrolyzing estrogen conjugates has been to boil the specimen for 1 hour with 15% hydrochloric acid. Brown has shown that, provided urine can be diluted 1:10 prior to this procedure, destruction of estriol, estradiol, and estrone can be
avoided (23). Such a dilution, however, is not usually suitable for the analysis of nonpregnancy urine because of the relatively large volumes required. Also, the presence of high glucose concentrations in urine has a destructive effect on the three estrogens when hot acid hydrolysis is performed without prior dilution (23, 24). It is reasons such as these which have prompted the use of enzymes ($\beta$-glucuronidase and sulphatase) for hydrolysis. However, enzyme hydrolysis holds certain disadvantages for the clinical chemistry laboratory. First of all, such a procedure is usually considerably more time-consuming than is conventional acid treatment; second, the use of some relatively crude enzyme preparations may interfere with estrogen color reactions (23); third, considerable inhibition of enzyme activity may occur in the urines of patients receiving various drugs, for example salicylates (25). In view of this, conventional hot-acid hydrolysis of diluted urine is the hydrolytic method of choice where "classic" estrogens are to be measured.

Other Chemical Methods

Other methods worthy of mention are those of Preedy (26) and of Ittrich (27, 28). Preedy's method employs column-partition chromatography with measurement of the separated estrogen fractions by fluorimetry of their sulfuric acid solutions. This type of fluorimetry, although much more sensitive than colorimetry, is also less specific. Because of this, its use in the hospital chemistry laboratory could create difficulties. This also applies to the method of Eberlein et al. (22). The Ittrich procedures introduce an interesting modification of the Kober reaction in which the estrogen is heated with diluted sulfuric acid and hydroquinone. The resulting chromogen is extracted into a solution of $p$-nitrophenol in chloroform (27) or, more conveniently, tetrabromoethane (28). Measurement can be made either colorimetrically or fluorimetrically. The procedure is more sensitive than the conventional Kober reaction and is also presumably more specific since the wave length of maximum light absorption, when colorimetry is used, is some $30 \text{m}\mu$ higher than is the case in the old Kober reaction. This being so, less interference will be observed from nonspecific chromogens absorbing at lower wave lengths. Fluorimetric measurement is also, in all probability, more specific than fluorescence induced by heating in sulfuric acid, since in the newer method it is associated with the more specific Kober-type reaction. It has been shown that the Ittrich method, in which the three estro-
gens are separated from each other and measured colorimetrically, is not superior to Brown’s procedure (29).

However, if fluorimetry is employed, the newer method may have certain advantages. The short procedures described by Ittirich are the ones most likely to make an impression in the hospital laboratory. Thus the new colorimetric procedure may be applied directly to the measurement of “total” estrogens in 0.1 ml. of pregnancy urine without any prior purification steps (27). Also, “total” estrogens are measurable, by the fluorimetric procedure, in 5 ml. of non-pregnancy urine with the minimum of purification steps (28). Many of such analyses can be done within a working day. Because of the lack of purification steps involved in these methods it must be assumed that great care will be required in measurements involving urines from patients on certain interfering medications. Also, the presence of glucose in some urines will still be a complicating factor.

References