

A RELIABLE METHOD FOR THE DETERMINATION OF URINARY ESTRIOL IN PREGNANCY

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SUMMARY

A reliable method for the estimation of urinary estriol in pregnancy is presented. This method is based upon acid hydrolysis, solvent partition, methylation and chromatography. Estriol is measured by colorimetry using the Kober reaction. All results are corrected for procedural loss by adding tritium-labelled estriol-16-glucosiduronate to the unhydrolyzed urine.

This method proves to be sufficiently specific, accurate and precise. One operator may complete six assays within one working day.

It is emphasized that a comprehensive recovery control which includes the initial hydrolytic procedure is essential if estriol determinations are to serve as a diagnostic aid in obstetrics.

INTRODUCTION

Estriol excreted in the urine of pregnant women serves as a valuable indicator of fetal well-being. The bulk of the maternal urinary estriol is produced by the fetoplacental unit¹. Estriol is readily transferred from the fetal compartment to the maternal circulation²⁻⁴. Maternal estriol metabolism is characterized by extensive conjugation, alteration of the estriol moiety to a limited extent, and a rapid urinary excretion, mainly in the form of estriol glucosiduronates⁵⁻⁷.

Fetal distress and/or placental insufficiency lead to prompt decrease in estriol production and excretion. Estriol determinations have therefore been considered helpful in timing delivery in pregnancies complicated by diabetes mellitus, hypertension and toxemia as well as placental insufficiency in order to prevent unnecessary prematurity and to reduce the risk of perinatal mortality⁸.

A number of estriol assays has been published. Many such methods are convenient, but only few are as reliable as the clinical importance demands. This paper presents a method for the determination of urinary estriol in pregnancy which is accurate as well as convenient.

EXPERIMENTAL

Trivial names

Estriol: estra-1,3,5(10)-triene-3,16 α ,17 β -triol; estriol-3-glucosiduronate: 16 α ,17 β -dihydroxy-estra-1,3,5(10)triene-3-yl- β -D-glucopyransiduronate; estriol-16-glucosiduronate: 3,17 β -dihydroxy-estra-1,3,5(10)-triene-16 α -yl- β -D-glucopyranosiduronate; estriol-3-methyl ether: 3-methoxy-estra-1,3,5(10)-triene-16 α ,17 β -diol.

Reagents

The reagents used were of analytical grade unless otherwise stated. All solvents except diethyl ether were distilled prior to use. Aluminum oxide (Al₂O₃ Woelm neutral, activity 1) was purchased from Alupharm Chemicals, New Orleans, La., U.S.A.; dimethyl sulfate, b.p. 75–77°/15 mm Hg, from Matheson, Coleman & Bell, East Rutherford, N.J., U.S.A.; [6,7-³H]estriol from New England Nuclear Corporation, Boston, Mass., U.S.A.; hydroquinone, extra pure, and silica gel G for thin-layer chromatography from E. Merck A.G., Darmstadt, Germany; and uridine-5'-diphosphoglucuronic acid from Sigma Chemical Company, St. Louis, Mo., U.S.A. Authentic estriol-16-glucosiduronate was a gift from Professor W. Allen, St. Louis, Mo., U.S.A.

Apparatus

The radioactive material was measured in a Packard Model 3375 Liquid Scintillation Spectrometer. Colorimetry was performed on a Beckman DU spectrophotometer. Tubes for a counter-current distribution (CCD) apparatus were obtained from AB. Laboratorietjänst, Stockholm, Sweden, and assembled to a hand-operated CCD machine.

Preparation of [³H]estriol-16-glucosiduronate

[6,7-³H]Estriol (E₃) was diluted to a specific activity (S. A.) of 5 μ C per μ g and purified by paper partition chromatography. Its radiochemical homogeneity was tested by crystallizing one aliquot to constant S.A. Radiochemically pure [³H]E₃ was incubated with an homogenate of human liver (autopsy material) and uridine-5'-diphosphoglucuronic acid according to the method of Slaunwhite *et al.*⁹. The resulting [³H]estriol-16-glucosiduronate (E₃-16Gl) was extracted from the incubation mixture and purified by a 24-transfer CCD in ethyl acetate 250, *n*-butanol 250, 0.2 *N* NH₄OH 500, where E₃-16Gl has a partition coefficient (*K*) of approximately 1, and by gel filtration on Sephadex G-25¹⁰. The radiochemical homogeneity of the [³H]E₃-16Gl fraction was assessed by subjecting one aliquot to a 24-transfer CCD in ethyl acetate 500, 0.1 *N* hydrochloric acid following the admixture of authentic E₃-16Gl. No ³H-labelled material could be dissociated from the carrier which was assayed by the Kober reaction according to Nocke's modification¹¹. Constant S.A. was obtained throughout the binomial distribution curve. Additional evidence that the conjugate prepared by this method is E₃-16Gl has been presented elsewhere².

Hydrolysis and solvent partition

Approximately 3000 counts/min of ³H-labelled E₃-16Gl are added to 1% or 0.5% of a total 24-hour urine collection from women less than 30 weeks and 30 or more weeks pregnant, respectively. Distilled water is added to increase the volume

to 50 ml, to which 10 ml of conc. hydrochloric acid is added. The specimens are boiled for 30 min under a reflux condenser. The hydrolyzate is cooled and extracted once with 120 ml of diethyl ether. The ether extract is washed twice with 25 ml of a mixture of 150 ml of 5 *N* NaOH and 1000 ml 8% (w/v) sodium bicarbonate and twice with 10 ml of distilled water. The ether is evaporated and its residue is partitioned between 50 ml of benzene-petroleum ether (1:1 v:v) and 50 ml of water containing 2% ethanol (v:v).

Methylation and chromatography

The aqueous phase is drained into an Erlenmeyer flask containing 4 ml of a solution of 22.5 g of boric acid in 100 ml of 5 *N* NaOH. One ml of dimethyl sulfate is added, the sample is vigorously shaken and kept for 30 min at 37°. Two ml 5 *N* NaOH and 1 ml dimethyl sulfate are added to repeat the methylation procedure.

After 20 to 30 min, 10 ml of 5 *N* NaOH and 2.5 ml of H₂O₂ (30%) are added. The methylation mixture is then extracted with 25 ml of benzene and the benzene extract is washed twice with 5 ml of distilled water and subjected to column chromatography according to Brown¹². The estriol-3-methyl ether (E₃-3Me) fraction is eluted with 20 ml of benzene containing 6% ethanol (v:v) into a small round bottom flask to be evaporated on a rotating evaporator.

Measurement

The residue of the E₃-3Me fraction is dissolved in 5 ml of methanol. A 2-ml aliquot serves to measure the ³H-labelled material recovered in the E₃-3Me fraction by liquid scintillation counting. Two 1-ml aliquots are subjected to the Kober reaction which is carried out according to the modification of Nocke¹¹. The optical density (*D*) of the developed colors is read at 480, 518 and 556 mμ and the corrected optical density (*D*_{corr}) is calculated by applying Allen's formula: $D_{\text{corr}} = 2D_{518} - (D_{480} + D_{556})$.

The amount of estriol excreted per 24 h is determined by the following formula, where *A* represents the number counts/min of ³H added, *R* the number of counts/min ³H recovered, *S* the sum of *D*_{corr} of the two 1-ml aliquots analyzed, *TV* the total 24-h urine volume, *AL* the aliquot assayed and *F* the factor to convert *D*_{corr} into μg of E₃:

$$E_3 = \frac{A \times S \times TV \times F}{R \times AL}$$

The factor *F* is calculated from calibration curves which are prepared with each batch of hydroquinone-sulphuric acid reagent¹¹.

RESULTS

Precision

The precision of the method was estimated by carrying out repeated estriol assays on 15-ml aliquots from a pool of pregnancy urine and calculating the standard deviation (S.D.) of the results from their mean. Eighteen determinations were performed. The mean value and its S.D. were 270 ± 12.3 μg, indicating a S.D. of ± 4.6%.

From 100 routinely performed estriol assays it was calculated that an additional error of $\pm 2.9\%$ (S.D.) would occur if estriol would be quantitated on the basis of a single colorimetric determination instead of performing Kober reaction and colorimetry on 2 aliquots per assay, as proposed in this paper.

Specificity

Aliquots of E_3-3Me fractions were pooled from a large number of routinely performed estriol assays. Aliquots of this pool were subjected to the Kober reaction. The spectrum of their Kober chromogens closely resembled that of authentic E_3-3Me .

Aliquots of the E_3-3Me pool were subjected to thin-layer chromatography using systems A (ethyl acetate 45–cyclohexane 45–ethanol 10) and L (chloroform 90–ethanol 10) of Lisboa¹³. After the plates had been subjected to the anisaldehyde–sulphuric acid color reaction performed according to Lisboa and Diczfalusy¹⁴, only one spot was detected exhibiting R_F values identical to those of authentic E_3-3Me (0.36 and 0.14 in systems A and L, respectively).

Another aliquot of the E_3-3Me pool was subjected to a 24-transfer CCD in carbon tetrachloride 500–methanol 200–water 300. Some 96% of the 3H -labelled as well as the Kober chromogenic material was distributed in a single binomial curve ($K = 0.6$) and a constant S.A. was obtained.

Accuracy

The accuracy of the method was determined by measuring the recovery of authentic E_3-16Gl added to the urine of a male subject known to excrete less than $0.1 \mu g$ of estriol per 10 ml of urine. One mg of estriol was added in the form of E_3-16Gl to 100 ml of urine and eight 10-ml aliquots were analyzed. The mean recovery and its S.D. were $100.5 \pm 2.2 \mu g$, indicating a virtually complete recovery.

From 300 estriol assays which were routinely performed by 2 operators it was calculated that the recovery of 3H -labelled material amounted to 59.6%. The S.D. was ± 11.5 , about $\pm 19\%$ of the mean recovery.

Convenience

Six samples may be completed by one operator within a working day. An accurate result will be obtained within less than 8 h following the completion of a 24-h urine collection. Relatively inexperienced operators will produce reliable determinations. Only 3 out of about 600 assays had to be repeated because less than 2% of the $[^3H]E_3-16Gl$ was found to be hydrolyzed and no Kober chromogenic material could be measured.

DISCUSSION

Out of the large number of methods to determine urinary estriol in pregnancy just a few assays utilize labelled estriol to correct for methodological loss. Only the method described by Jaffe and Levitz¹⁵ employs $[^3H]E_3-16Gl$ to account for losses due to incomplete hydrolysis. Approximately 95% of all the estriol excreted in pregnancy urine was found to be present in the form of glucosiduronates, mainly as E_3-16Gl^7 . Labelled E_3-16Gl therefore appears to be the most suitable recovery standard rendering this method close to 100% accurate. Incomplete hydrolysis has been

encountered in routinely performed estriol assays. Without the addition of labelled E_3 - $16G1$, falsely and even alarmingly low estriol values would have been reported.

The method presented is reasonably precise. Its precision and accuracy are not only due to the utilization of labelled E_3 - $16G1$, but are also achieved by the reliability of the Kober reaction when performed following methylation and chromatography, two essential steps of Brown's original procedure¹². The method of Jaffe and Levitz¹⁵ does not include methylation and chromatography and is therefore shorter and more convenient. The latter method, however, employs Ittrich's extraction of the Kober chromogens¹⁶ and fluorimetry to compensate for its shorter purification procedure. Although the procedure of Jaffe and Levitz appears to be reliable, there still remains the possibility of occasional quenching of fluorescence which would result into falsely low estriol measurements.

A somewhat lower precision is obtained if only one aliquot per assay is measured colorimetrically. It remains a matter of opinion whether or not the additional error of $\pm 2.9\%$ (S.D., $n = 100$) warrants the efforts of performing routinely Kober reaction and colorimetry in duplicates. It appears advisable, however, to measure the Kober chromogens in duplicates whenever small amounts of E_3 - $3Me$ are likely to be encountered as in cases of suspected intra-uterine fetal death.

Adequate specificity has been achieved. When additional steps of Brown's original procedure¹² were omitted in this method, it became less specific as evidenced by thin-layer chromatography. At its present length the proposed method remains reasonably convenient. One operator may perform 6 assays within a normal working day and no more than average skills are required.

The normal range of estriol excretion is large and may vary significantly in the same subject from day to day. As Klopper⁹ indicated "it is essential that this element of physiological variability should not be farther compounded by methodological error if estriol assays are to be of clinical value". Our method, without its recovery control, would measure approximately 60% of the true estriol excretion with a S.D. ($n = 300$) of $\pm 19\%$ of the mean recovery. It is obvious how low the true estriol excretion must drop before it becomes significant if measured with one of the many short and uncorrected assays.

The method presented has not yet been employed to measure the urinary excretion of estriol in a large number of uncomplicated pregnancies. Klopper and Billewicz¹⁷ have measured estriol excretion in normal pregnancies by using the method of Klopper and Wilson¹⁸, which is comparable to this method except that it does not utilize a labelled internal standard to correct for methodological losses. Our normal values were found to be within the range of those reported by Klopper and Billewicz¹⁷ if their values are corrected for an average recovery of 80%.

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