THE USE OF SILICA GEL IMPREGNATED GLASS FIBER SHEETS
IN PLASMA ESTRIOL ASSAYS

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ABSTRACT

The fluorimetric quantitation of nanogram amounts of estriol
extracted from plasma is sometimes impaired by impurities which
interfere with fluorescence. Instant thin-layer chromatography on
silica gel impregnated glass fiber sheets (ITLC-SG) removes such
impurities originating from extracts as well as from solvents used
in the course of analysis. This convenient, rapid and effective
chromatographic procedure does not introduce interfering substances
by itself. With the utilization of ITLC, plasma estriol assays proved
to be precise, specific and accurate.

The first relatively simple and generally applicable method
for serial clinical determinations of plasma estriol (3) concentrations
during the second half of human pregnancy was published by Nachtigall
et al. (4). With this method, fluorimetry has proven to be a practical
way of quantitating nanogram amounts of estriol derived from plasma
extracts. In attempting to adapt our method of measuring urinary estriol in pregnancy (5) to the determination of plasma estriol in the second half of gestation, unsatisfactory precision and accuracy were encountered although methylation, additional solvent partition and chromatography on alumina were used to remove impurities which not infrequently interfere with fluorescence. Such impurities may arise from plasma or other biological fluids as well as from solvents used during the extraction and purification procedures. A purification step appeared necessary which would effectively remove these impurities and not introduce interfering substances inherent in many chromatographic procedures.

This report describes the use of instant thin-layer chromatography (ITLC) on silica gel impregnated glass fiber sheets to improve specificity, accuracy and precision in the measurement of plasma estriol.

EXPERIMENTAL

Materials. Solvents and reagents were of analytical grade unless otherwise stated. All solvents except diethyl ether (USP, Mallinckrodt Chemical Works) were distilled prior to use. Benzene was shaken for 3 hours with concentrated sulfuric acid, washed successively with distilled and demineralized water, a saturated sodium carbonate solution and again with water and was finally distilled over sodium hydroxide pellets. P-nitrophenol (MP 113–114°C) was purchased from Matheson, Coleman & Bell, East Rutherford, N.J., hydroquinone (extra pure, Merck) and sulfuric acid (ultra pure, Merck) from Brinkmann Instruments, Westbury, N.Y., and silica gel impreg-
nated glass fiber sheets (ITLC-SG) from Gelman Instrument Company Ann Arbor, Michigan. Estriol-6,7-3H was purchased from Amersham/Searle, DesPlaines, Ill., and uridine-5'-diphosphoglucuronic acid from Sigma Chemical Company, St. Louis, Mo. All non-labeled steroids were purchased from Steraloids, Pawling, N.Y.

Apparatus. The radioactive material was measured in a Packard Model 3320 liquid scintillation spectrometer. Fluorimetry was carried out in an Aminco-Bowarann spectrofluorimeter, used without filters.

Preparation of 3H-estriol-16-glucosiduronate. Estriol-6, 7-3H with a specific activity (S.A.) of 8 µCi/µg was purified by paper partition chromatography (benzene:methanol:water, 2:1:1). Its radiochemical purity was tested by crystallizing an aliquot to constant S.A. Radiochemically pure estriol-6,7-3H was incubated with an homogenate of human liver (autopsy material) and uridine-5'-diphosphoglucuronic acid according to the method of Slaunwhite et al. (6). The resulting 3H-estriol-16-glucosiduronate was extracted from the incubation mixture, purified by countercurrent distribution and gel filtration on Sephadex G-25 and examined for radiochemical homogeneity as previously described (5).

Preparation of non-labeled estriol-16-glucosiduronate. Estriol-16-glucosiduronate (E3-16G1) was prepared similarly by incubating 100 mg of crystalline estriol (E3) with 1 gm of uridine-5'-diphosphoglucuronic acid in 200 ml of 0.1 M phosphate buffer, pH = 7.4, containing a homogenate of 20 gm of human liver. The E3-16G1 obtained from the incubation mixture was subjected to three 24-transfer countercurrent distributions in ethyl acetate:n-butanol:0.2 N NH4OH (1:1:2), in ethyl acetate:0.1 N HCl (1:1) and in n-butanol:water (1:1) with partition coefficients of 1.0, 1.4 and 1.2, respectively. The material obtained was recrystallized from acidified n-butanol. It was dissolved and stored in 80% ethanol following neutralization with NH4OH. The yield was 27.2%. It has been proven that the estriol conjugate obtained under the above incubation is indeed E3-16G1 (7).

Hydrolysis and extraction procedure. The method used for plasma estriol determinations was essentially that of Nachtigal et al. (4) as modified by Levitz and Bassett (8). One ml of plasma (EDTA is used as the anticoagulant) was added into a heavy-wall centrifuge tube of 35 ml capacity which may be closed with a teflon-lined screw cap. About 20,000 DPM of 3H-E3-16G1 was added in 50 µl of 80% ethanol. Two ml of distilled water were added together with 0.60 ml of concentrated hydrochloric acid. The tube was closed and kept in boiling water for
30 minutes. The hydrolysate was cooled, 1 ml of water added and the mixture extracted twice with 10 ml of diethyl ether, using a Vortex Jr. mixer for extraction. Centrifugation was then performed to effect rapid separation of the ether from the water phase. The ether phases were combined in another centrifuge tube, washed with 3 ml of a sodium bicarbonate buffer prepared from 150 ml of 5 N sodium hydroxide and 1000 ml 8% (w/v) sodium bicarbonate (9) and finally washed twice with 2 ml of distilled water. The ether phase was evaporated and its residue dissolved in 0.08 ml of ethanol and 4 ml of distilled water. This was extracted with 4 ml of a benzene:n-hexane mixture (1:1). The organic phase was discarded. The aqueous phase was extracted twice with 4 ml of ether, which was transferred into a conical test tube for evaporation in a warm water bath.

Instant thin-layer chromatography. The residue was dissolved in a few drops of methanol and spotted on ITLC-SG sheets applying 5 samples per sheet (20 x 20 cm) adjacent to 3 µg of authentic E3. The ITLC-SG sheets were suspended in an unlined thin-layer chromatography jar and developed with chloroform containing 1% ethanol (v:v). The spot of authentic E3 was visualized by the Folin reaction (10) and its Rf utilized to cut out 3 cm wide zones of the sheet containing the E3. These zones were eluted with 2.5 ml of methanol.

Quantitation. In order to correct for procedural loss, 0.5 ml of the eluate was taken for liquid scintillation counting, while 1.5 ml was used for fluorimetry. This was added to glass-stoppered centrifuge tubes which contained 1 ml of 2% (w:v) hydroquinone in ethanol. The tubes were evaporated under nitrogen which had been passed through conc. sulfuric acid and soda lime. Sixty-five per cent ultra-pure sulfuric acid was used to develop the Kober chromogens by boiling the stoppered tubes for 40 minutes. Following the addition of 1.5 ml of glass-distilled water, the fluorescent material was extracted into chloroform containing 1% ethanol (v:v) and 2% p-nitrophenol (w:v) according to Mahesh's modification (11) of Ittrich's procedure (12). Under these conditions using tubes cleaned with sulfuric acid and assaying not more than 12 tubes at a time (13), calibration curves were well reproducible. A linear regression line with a coefficient of correlation of 0.99 was obtained in 4 successive calibrations with amounts ranging from 5-100 ng of estriol.

RESULTS

Separation of estrogens on ITLC. The chromatographic behavior of 9 estrogens on ITLC-SG has been studied in several solvent systems.
Since chloroform was the solvent used to extract the fluorescent material from the sulfuric acid/water mixture for fluorimetry, chloroform was preferred for the development of the ITLC-SG sheet in this assay, since its impurities would be least likely to interfere with fluorescence. Solvent system No. 2 containing 1% ethanol in chloroform was used in plasma estriol assay.

Table 1.

R_F values for 9 estrogens on ITLC-SG in the following solvent systems: 1) 2% ethanol in chloroform; 2) 1% ethanol in chloroform; 3) chloroform; 4) cyclohexane:chloroform (3:7); 5) cyclohexane:chloroform (2:3); 6) ether:benzene (1:9) and 7) ethyl acetate:cyclohexane (1:9). Each figure represents the mean R_F of 3 chromatographic runs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent Systems</th>
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</thead>
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<tr>
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<td>2-methoxy-E_1</td>
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</tr>
<tr>
<td>E_1</td>
<td>.99</td>
</tr>
<tr>
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<td>16-epi-E_3</td>
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</tr>
<tr>
<td>E_3</td>
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Precision. The precision of the procedure was estimated by having one technician carry out 14 replicate assays on two consecutive
days on a pool of plasma collected from women in the second half of
gestation and by calculating the standard deviation (S.D.) of the results
from their mean. The mean concentration and its S.D. were 20.4 ± 1.2
µg/100 ml of plasma, indicating a coefficient of variation of ± 5.7%.

Snedecor (14) suggested a method of obtaining an estimate of
precision in which the S.D. is calculated from the difference between
the two results of duplicate determinations in a series of assays. Forty-
seven duplicate determinations were carried out and the S.D. was calculated
as suggested by Snedecor (14) except that the difference between the
results of each pair of assays was expressed in per cent of the mean
of the two results. The coefficient of variation in this series of
duplicate assays was found to be ± 2.9%.

Accuracy. The accuracy was determined by measuring the
recovery of estriol added to plasma in the form of E\textsubscript{3}-16G1. Twenty-
four samples were analyzed after amounts of 250, 333 and 500 ng of
estriol were added to 1 ml aliquots of pooled male plasma. The results
are indicated in Figure 1. Using the internal standard to correct for
procedural loss, virtually all of the estriol added as the 16-glucosiduronate
was accounted for.

Sensitivity. When pooled male plasma was analyzed in 7 replicate
assays, a reading was obtained, which after correction for procedural
Fig. 1. Recovery of estriol added to plasma in the form of estriol-16-glucosiduronate.

loss, amounted to an equivalent of $1.1 \pm 0.3 \mu g$ of estriol per 100 ml of plasma. When 8 water blanks were assayed in the same manner, comparable results were obtained. Thus the proposed method is not suitable for plasma estriol assays prior to the 20th week of gestation, when estriol reaches a plasma concentration of about 4 $\mu g/100 \text{ ml}$. 
Plasma estriol levels in pregnancy. Plasma estriol levels were determined once or several times at biweekly intervals in 40 healthy pregnant women with uncomplicated pregnancies (Fig. 2). Since plasma levels of estriol are subject to diurnal changes (15), all specimens were drawn between 8 and 11 a.m. The measurements agreed well with those reported by Nachtigall et al. (4) reaching levels in excess of 40 \( \mu \text{g}/100 \text{ ml} \)

![Graph showing plasma estriol concentrations](image)

Fig. 2. Plasma estriol concentrations from 40 normal women during the second half of gestation.
at term. Although the normal increase in urinary estriol excretion was not always paralleled by a concomitant rise in plasma concentrations, plasma estriol levels generally appeared to rise most rapidly during the last 5 or 6 weeks of gestation as did the urinary estriol excretion.

DISCUSSION

The steady increase in estriol biosynthesis, plasma concentration and urinary excretion during human pregnancy is utilized for evaluating fetal well-being (16). The bulk of maternal plasma and urinary estriol is produced by fetus and placenta (17) and readily transferred to the maternal circulation (7, 18, 19). Estriol metabolism in the mother consists mainly of conjugation while the estriol moiety remains largely unchanged (20-22). The determination of urinary estriol excretion has proven to be a valuable diagnostic adjunct in obstetrics but is burdened with the inconvenience, delay and frequent inaccuracy in collecting a 24-hour urine specimen. Much interest has therefore been focused on plasma estriol assays.

The fluorimetric quantitation of nanogram amounts of estriol has the advantages of convenience, relative specificity and sufficient sensitivity. However, accuracy and precision are sometimes inadequate due to impurities which enhance or, more frequently quench, fluorescence. Such impurities are extracted from biologic specimens and not dissociated
from estriol during purification procedures. Additional impurities are often introduced with solvents and reagents. In an attempt to adapt our method for urinary estriol determination (5) to plasma estriol assay we used methylation, benzene/water partition and chromatography on alumina columns to further purify our plasma extracts. Precision and accuracy, however, were still inadequate and it was found that the solvents and reagents involved in this step contributed impurities which quenched fluorescence.

The use of silica gel impregnated glass fiber sheets for the separation of phenolic steroid sulfate has been described by Payne and Mason (23). These ITLC sheets, when developed in chloroform and eluted with methanol, did not contribute enhancing or quenching impurities to the fluorimetric quantitation of estriol.

The ITLC used in this assay separates estriol from estrone, estradiol and other estrogens. Thus the partition between water containing 2% ethanol and benzene:n-hexane (1:1) may seem superfluous since it was designed by Brown (9) to separate estriol from estrone and estradiol. This partition, carried out twice in the method of Nachtigall et al. (4), has been retained in the form of one partition in order to remove lipids prior to ITLC. In our experience, lipids interfered with the migration of estrogens on ITLC-SG which provides good resolution but has limited capacity.
The precision of the method is acceptable as is its accuracy, the latter being assessed in regard to the recovery of $E_3$-16G1. Although Ertel et al. (24) presented evidence that there was no difference in the measurement of plasma estriol whether tritiated $E_3$, $E_3$-16G1 or $E_3$-3S was used as an internal standard, our method utilizes $^3$H-estriol-16-glucosiduronate as suggested by Levitz and Bassett (8). More recently, the plasma estriol method described in this paper has been used in our laboratory for the measurement of estriol levels in amniotic fluid.

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REFERENCES

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3. The following trivial names and abbreviations are used in this paper: estrone ($E_1$) = 3-hydroxyestra-1,3,5(10)-trien-17-one; 2-methoxyestrone (2MeO-E$_1$) = 2-methoxy-3-hydroxyestra-1,3,5(10)-trien-17-one; 17β-estradiol ($E_2$) = estra-1,3,5(10)-triene-3,17β-diol; 16α-hydroxyestrone (16αOH-E$_1$) = 3,16α-dihydroxyestra-1,3,5(10)-trien-17-one; 16β-hydroxyestrone (16βOH-E$_1$) = 3,16β-dihydroxyestra-1,3,5(10)-trien-17-one; 16-ketoestradiol-17β (16-oxoE$_2$) = 3,17β-dihydroxyestra-1,3,5(10)-trien-16-one; 16-epiestriol (16epiE$_3$) = estra-1,3,5(10)-
triene-3,16β,17β-triol; 17-epiestriol (17epiE3) = estra-1,3,5(10)-triene-3,16α,17α-triol; estriol (E3) = estra-1,3,5(10)-triene-3,16α,17β-triol; estriol-16-glucosiduronate (E3-16G1) = 3,17β-dihydroxyestra-1,3,5(10)-triene-16α-yl-β-D-glucopyranosiduronate; estriol-3-sulfate (E3-3S) = 16α,17β-dihydroxyestra-1,3,5(10)-triene-3-yl-sulfate; estriol-3-sulfate-16-glucosiduronate (E3-SG1) = 17β-hydroxyestra-1,3,5(10)-triene-3-yl-sulfate-16α-yl-β-D-glucopyranosiduronate.


