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ESTRIOL CONJUGATES IN BODY FLUIDS IN LATE HUMAN PREGNANCY*

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SUMMARY

A new method was developed for the assay of estriol (E_3) conjugates in amniotic fluid, urine and plasma of third trimester human pregnancy. Tritiated estriol-3-sulfate (E_3-3S), estriol-16-glucosiduronate (E_3-16G) estriol-3-glucosiduronate (E_3-3G) and estriol-3-sulfate-16-glucosiduronate (E_3-3S-16G) are added to the fluid. The conjugates are separated as their triethylammonium salts on Sephadex LH-20. Each conjugate is hydrolyzed enzymatically and the estriol is purified by solvent extractions. The estriol is quantitated by radioimmunoassay. In the amniotic fluid E_3-16G and E_3-3S-16G comprised about 75% of the total E_3 conjugates. In normal pregnancy the E_3-16G/E_3-3S-16G ratio increased markedly with gestational age. In Rh-isoimmunization disease the ratio increased less dramatically and the patterns were erratic. In the urine E_3-16G predominated (70–80%) whereas in the plasma E_3-3S-16G comprised about 45% and E_3-16G about 25% of the total. The concentrations of E_3-3S and E_3-16G were variable. The renal clearances in ml/min of the E_3 conjugates measured in 2 subjects are as follows: E_3-16G, 343–508 (similar to that of p-aminohippuric acid); E_3-3G, 64–149; E_3-3S, 13–34; and E_3-3S-16G, 21–29. These methods appear applicable to the study of a variety of conditions in pregnancy in which pathology may be reflected in abnormal profiles of E_3 conjugates.

INTRODUCTION

The concentration of estriol in body fluids rises impressively throughout human gestation. Focusing on the urine, plasma or amniotic fluid, numerous investigators have related estriol (E_3)‡ levels to fetal-placental health [1–6]. However in each fluid estriol is found principally as four conjugates: estriol-3-sulfate (E_3-3S), estriol-16-glucosiduronate (E_3-16G), estriol-3-glucosiduronate (E_3-3G) and estriol-3-sulfate-16-glucosiduronate (E_3-3S-16G) [7–9]. The concentration of each conjugate in body fluids appears to be controlled by complex metabolic and transport mechanisms. Consequently, a more complete understanding of the relationship of estriol with diseases of pregnancy may derive from the knowledge of the levels of these conjugates in normal and abnormal pregnancy. The purposes of the present studies were twofold; (1) To develop more convenient methodology for the measurement of the four estriol conjugates in fluids of late human pregnancy, and (2) Apply the methodology to specific clinical situations. Of particular interest were (a) the concentration of estriol conjugates in the amniotic fluid in relation to stage of gestation in normal gestation and in pregnancy complicated by Rh-isoimmunization and (b) the renal clearance of the individual conjugates at term in normal pregnancy.

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‡ The following abbreviations and trivial names are used in this paper: E_3-3S = estriol-3-sulfate,16α,17β-dihydroxyestra-1,3,5(10)-trien-3-yl-sulfate; E_3-16G = estriol-16-glucosiduronate, 3,17β-dihydroxyestra-1,3,5(10)-trien, 16α-yl-β-D-glucopyranosiduronate; E_3-3G = estriol-3-glucosiduronate, 16α,17β-dihydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; E_3-3S-16G = estriol-3-sulfate-16-glucosiduronate, 17β-hydroxyestra-1,3,5(10)-trien-3-yl-sulfate-16α-yl-β-D-glucopyranosiduronate; PAH = p-aminohippuric acid; E_3-6-CMO = estriol-6-(O-carboxymethyl) oxime; BSA = bovine serum albumin.

EXPERIMENTAL

Subjects and fluids

Amniotic fluid samples were obtained by amniocentesis. The procedure was performed on normal subjects in whom it was necessary to obtain samples for the determination of lecithin to sphingomyelin (L/S) ratios [10]. Amniotic fluids were obtained from subjects with Rh-isoimmunization for optical density analysis [11]. Specimens were stored at −17°C until analyzed for the estriol conjugates. Plasma and urine samples were obtained from women who volunteered to undergo renal clearance tests. Each patient was about to undergo repeat cesarean section and was apparently normal. A Foley catheter was inserted for the collection of urine and an intravenous infusion was started. The patient received p-aminohippuric acid (PAH) and inulin for the determination respectively of tubular secretion rate and glomerular filtration rate [12, 13]. Blood samples were drawn at time zero and then at three 40 min intervals, during which time three urine samples were collected. Plasma and urine were stored at −17°C until analyzed for estriol conjugates, PAH and inulin. The renal clearance of each substance analyzed was calculated [14], corrected for standard surface area.

Radioactive estriol conjugates

The four radioactive estriol conjugates were synthesized from [2,4,6,7-^{3}H]-estriol 110 Ci/mmol (New England Nuclear Co., Boston, Mass.) and each was

purified by gradient elution chromatography on Celite [9].

Separation of estriol conjugates

Amniotic fluid, plasma and urine were processed in the same way. About 2000 c.p.m. of each estriol conjugate were added to 0·2 to 1·0 ml of the fluid to be analyzed. The conjugates were converted to their triethylammonium salts and chromatographed on Sephadex LH-20 (Pharmacia, Piscataway, N.J.) as described in detail previously [15, 16]. The system ethylene chloride with increasing concentrations of t-butanol was used to elute the conjugates in the order: E_3-3S-16G, E_3-3S, E_3-16G. Finally, E_3-3G was eluted with methanol.

Hydrolysis of conjugates and purification of estriol

Each conjugate was hydrolyzed with Glusulase [9]. The estriol was extracted with ether and following evaporation the residue was partitioned between benzene–hexane–water [1:1:2]. The water phase was extracted with ether. The ether was evaporated and 2·0 ml of distilled ethanol was added. An aliquot (0·2 ml) was used to monitor recoveries and other aliquots were submitted to radioimmunoassay.

Radioimmunoassay

Two different types of radioimmunoassay were used in this study. The amniotic fluid was assayed using a glass-bound antibody, whereas the urine and plasma were assayed by the conventional Dextran-coated charcoal procedure.

Glass-bound antibody method

Arylamine glass was prepared from controlled pore glass, 550 Å pore dia., 1–4 μm average dia. (generously supplied by Corning Glass Works, Corning, N.Y.). The glass was diazotized, reacted with ovine anti-E_3 serum and prepared for use as described [15]. The antisera was generously supplied by Dr. W. G. Kelly and Dr. E. Gurpide. The E_3 was rendered antigenic by linkage to bovine serum albumin via the 16,17-dihemisuccinate [17]. Details of this solid-phase radioimmunoassay have been published [15].

Dextran-coated charcoal method

Rabbit antisera produced from estriol-6-(O-carboxymethyl) oxime (E_3-6-CMO) linked to bovine serum albumin [18] (generously supplied by Dr. U. Goebelsmann) was used in the assay of plasma and urine. The procedure utilized dextran-coated charcoal to separate bound from unbound E_3, essentially as described [19].

RESULTS

The assay procedure

Figure 1 shows that the four estriol conjugates are readily separated as their triethylammonium salts on a Sephadex LH-20 column. The ensuing steps of enzyme hydrolysis and purification of E_3 by solvent partition are similar to those described [9], and require no further comment. The overall recovery of internal tracer ranged from 30–70%.

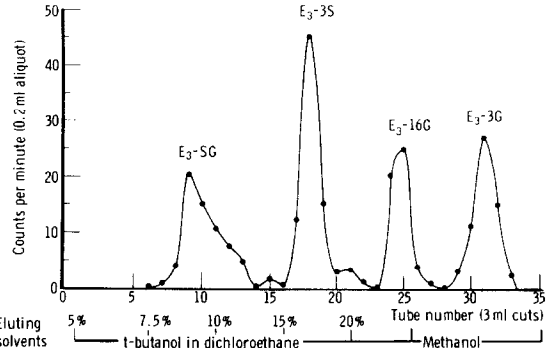

Fig. 1. Profile of estriol conjugates eluted from Sephadex LH-20. The column was 22 × 1·4 cm and contained 9 g of Sephadex. The order of elution is estriol-3-sulfate-16-glucosiduronate (E_3-SG), estriol-3-sulfate (E_3-3S), estriol-16-glucosiduronate (E_3-16G), and estriol-3-glucosiduronate (E_3-3G).

E_3 derived from amniotic fluid was assayed using a glass-bound antibody. The standard curve indicated a useful range of assay up to 1500 pg. However, since the water blank was about 40 pg, assays resulting in values of less than 500 pg were repeated with greater aliquots. The precision is excellent, the average deviation rarely exceeding 5%. Tests with a variety of phenolic and non phenolic steroids indicate that the glass-bound antibody has essentially the same specificity as the native antibody. The accuracy of the method was evaluated by comparing the results obtained by the procedure described here with those obtained by the fluorometric method described earlier [9]. The values usually differed by about 10%. In the initial phase of the study the urine and plasma samples were also assayed by the glass-bound antibody method. However, male plasma blanks amounted to 0·01 to 0·015 μg/ml, a fairly high fraction of E_3 conjugates in some plasmas. Assay by the dextran-coated charcoal (DCC) method using antisera to E_3-6-CMO-BSA produced a male plasma blank in the range of 0·001 μg/ml for each E_3 conjugate which is insignificant. The specificity and sensitivity of the DCC method render it more attractive for the assay of low levels of E_3 conjugates in the plasma. For uniformity the urine was assayed in the same way in the renal clearance studies.

Estriol conjugates in amniotic fluid

Twenty amniotic fluid samples taken between 32 and 40 weeks of gestation were analyzed using the glass-bound antibody. In μg/100 ml the ranges for the 4 estriol conjugates were as follows: E_3-3S, 2·9–34·8; E_3-16G, 17·3–58·5; E_3-3G, 2·0–13·7; E_3-3S-16G, 7·7–31·9. The only consistent picture is the rise in the E_3-16G to E_3-3S-16G ratio as pregnancy progresses. (Table 1). These two conjugates usually comprised more than 75% of the total. There were no consistent patterns with respect to E_3-3S and E_3-3G.

Table 1.

E_3-16G/E_3-3S-16G ratios in amniotic fluid in normal pregnancy and in pregnancy complicated by Rh-isoimmunization

Weeks Gestation	Normal	Rh Disease
28-31		0.7 ± 0.3 (2)
32-34	1.1 ± 0.2 (8)	0.8 ± 0.5 (4)
35-37	1.9 ± 0.4 (9)	1.5 ± 1.0 (10)
38-40	2.4 ± 0.5 (6)	

The number of cases are given in parentheses. The average deviations are also shown.

Table 2.

E_3-16G/E_3-3S-16 ratios in serial samples of amniotic fluid from normal (N) and Rh-isoimmunized (Rh) subjects

Subject	Status	Sample Number 1	2	3	4
MO	N	1.4 (33)	3.2 (36)		
GO	N	0.7 (32)	1.3 (35)		
RP	N	0.8 (34)	1.8 (36)		
EG	N	1.0 (34)	1.2 (35)	1.8 (36)	
CH	N	1.6 (36)	2.3 (38)		
CV	Rh	0.4 (28)	1.5 (32)	4.3 (35)	3.0 (36)
FF	Rh	1.2 (29)	1.0 (34)		
DJ	Rh	0.3 (31)	1.3 (32)	0.2 (36)	
HC	Rh	0.5 (34)	1.5 (35)	0.4 (37)	
FR	Rh	1.5 (36)	1.2 (37)		

Weeks of gestation are shown in parentheses.

Because amniocentesis was dictated by clinical consideration the 16 samples taken from patients with Rh-isoimmunization included 10 between 35 and 37 weeks of gestation. This is the only stage where comparisons could be made between the normal and diseased states. The E_3-16G/E_3-3S-16G ratios were 1·9 and 1·5 respectively which is not statistically different. However, in five cases from each group at least two samples were obtained. The results in Table 2 indicate that in the five normal cases the E_3-16G/E_3-3S-16G ratio increased as gestation progressed. On the contrary in each Rh patient the ratio dipped at least once.

Studies with maternal plasma and urine

These studies were designed to measure the renal clearance rates of each estriol conjugate. Comparisons were made with the renal clearance rates of PAH and inulin. The experimental design permitted observations at three consecutive 40 min periods. In Subject A, samples of plasma and urine were assayed by the two radioimmunoassay methods. The results in Table 3 show many values in good agreement, but there are some serious discrepancies, particularly in the plasma samples. The results obtained in Subject C are shown in Table 4. Similar to the results in Subject A, except for E_3-3G, the fluctuations of the E_3 conjugates in the plasma were within 25%. The calculated renal clearances for the E_3 conjugates, PAH and inulin in the two subjects, corrected for surface area are shown in Table 5. It is apparent that the clearance of E_3-16G approaches that of PAH. The values for E_3-3G are similar to those of inulin whereas the clearances of E_3-3S and E_3-3S-16G are much less than that of inulin.

Table 3.

Concentration of estriol conjugates in plasma and urine in human pregnancy at term: Comparison between radioimmunoassay by dextran-coated charcoal (DCC) and glass-bound (GB) antibody methods; Patient A.

Sample	Estriol Conjugate E_3-3S DCC	E_3-3S GB	E_3-16G DCC	E_3-16G GB	E_3-3G DCC	E_3-3G GB	E_3-3S-16G DCC	E_3-3S-16G GB
P-0	0.020	0.027	0.052	0.031	0.028	0.029	0.063	0.113
P-1	0.021	0.017	0.043	0.032	0.032	0.021	0.074	0.066
P-2	0.017	0.023	0.036	0.062	0.016	0.030	0.053	0.142
P-3	0.024	0.020	0.056	0.056	0.033	0.011	0.079	0.082
U-1	0.315	0.349	9.85	8.60	1.83	1.79	0.94	1.04
U-2	0.445	0.532	11.2	11.7	2.88	2.00	1.24	1.26
U-3	0.472	0.722	11.9	10.5	2.18	2.35	1.18	2.22

Values are in ug/ml
Plasma was drawn at time zero (P-0) and at 3 successive 40 minute periods. Urines (U) were collected during these 3 periods. Details of the 2 radioimmunoassays are given in the text.

Table 4.

Concentration of estriol conjugates in plasma and urine of patient C at term

Sample	Estriol Conjugate E_3-3S	E_3-16G	E_3-3G	E_3-3S-16G
P-0	0.060 (18)	0.052 (16)	0.047 (14)	0.173 (52)
P-1	0.048 (19)	0.065 (25)	0.016 (6)	0.129 (50)
P-2	0.066 (24)	0.049 (18)	0.034 (12)	0.129 (46)
P-3	0.054 (21)	0.061 (23)	0.025 (10)	0.122 (47)
U-1	1.58 (3)	45.2 (79)	3.36 (6)	6.73 (12)
U-2	1.36 (3)	33.7 (80)	2.50 (6)	4.60 (11)
U-3	0.92 (3)	27.9 (79)	2.29 (7)	4.09 (12)

Values are in μg/ml.
Value in parenthesis indicates the percent contribution of the conjugate to the total.
Radioimmunoassay was by the dextran-coated charcoal method as described in the text.

DISCUSSION

Since the pioneering studies of Troen *et al.*[20] which indicated the complex nature of estriol conjugates in the amniotic fluid, several investigations have concentrated on streamlining the methodology and gathering more accurate data. Goebelsmann and Work[21] combined separation of the conjugates by countercurrent distributions with fluorometric measurement for their assays. The method of Tikkanen and Adlercreutz[22, 23] utilized fractionation by sequential chromatography on Sephadex (3 columns) and quantitation by gas liquid chromatography. Our original method which was the simplest exploited the separation of the four conjugates on a single Celite column and assay by fluorometry [9].

This paper presents two improvements which render our method even more attractive. First, the four conjugates are separated on a relatively short Sephadex LH-20 column, which permits the processing of four samples in a working day. Secondly, quantitation by radioimmunoassay presents the advantage of sensitivity so that 0·2 ml samples usually suffice. Two radioimmunoassay procedures were employed in this study. The first involved the use of a glass-bound antibody, which permits the separation of bound from unbound antigen by simple centrifugation. The method seems highly suited for the amniotic fluid studies.

On application of the method to the study of plasma E_3 conjugates it was found that the male plasma blank was 0·01 to 0·02 μg/ml, an unacceptably high value. Whether the problem is inherent in the nature of solid-phase support *per se* or with the limited specificity of an antibody produced from E_3-16,17-dihemisuccinate-BSA cannot be ascertained. However, the use of a more specific antibody produced against E_3-6-CMO-BSA reduced the blank tenfold. The decision to employ the Dextran-coated charcoal method was dictated by the limited amount of antisera available. About 85% antigenicity is usually lost on linking the antisera to glass.

The most significant result of the amniotic fluid studies is the increase in the E_3-16G/E_3-3S-16G ratio as gestation proceeds. Furthermore the increase appears to accelerate at about 35 weeks, around the time of fetal lung maturation, although no link between these phenomena is implied. The data in Table 1 suggest that at comparable stages of gestation the E_3-16G/E_3-3S-16G ratios are lower in pregnancy complicated by Rh-isoimmunization than in normal pregnancy. The highly unusual pattern of subject CV (Table 2) and the low number of observations combined to render the differences statistically not significant. However, analyzing the subjects studied serially, it is apparent that the Rh group displays a pattern different from the normal. Parenthetically a similar conclusion was reached by Goebelsmann and Work[21] on the basis of very fragmentary evidence. Furthermore, the studies of Tikkanen and Adlercreutz[23] are corroborative, although these authors did not discuss their data in terms of the E_3-16G/E_3-3S-16G ratio.

There are distinct differences between the maternal plasma and urine in the composition of the E_3 conjugates. In the urine E_3-16G predominated, but in the plasma the concentration of E_3-3S-16G was highest. These differences are reflected in the renal clearances shown in Table 5. The renal clearance of E_3-16G approximates that of PAH, strongly indicating a tubular secretion for this E_3 conjugate. This was first suggested by Brown *et al.*[24] but not clearly demonstrated until the present study. In some instances the renal clearance of E_3-16G actually exceeded that of PAH. A contribution to the calculated renal clearance of E_3-16G of unknown magnitude, but not truly representing renal clearance, is that part of blood-borne unconjugated E_3 which is glucuronidated in the kidney and excreted [25]. The behavior of E_3-3G is somewhat puzzling. In patient A the value clearly exceeded that of inulin but in patient C the values for E_3-3G clearance were slightly less. Further studies are indicated. The low renal clearances of E_3-3S and E_3-3S-16G can be attributed to the greater protein binding of these conjugates [8].

The simplified methodology described in this paper should stimulate inquiries into aberrations in E_3 conjugates in relation to diseases of pregnancy. Abnormally low concentrations of E_3-3G in the urine of women presenting with cholestasis of pregnancy has been documented [26]. The studies described here

Table 5.

Renal clearance of estriol conjugates, p-aminohippuric acid (PAH) and inulin in term human pregnancy during 3 successive 40 min periods in 2 subjects.

Subject	Period	E_3-3S	E_3-16G	E_3-3G	E_3-3S-16G	PAH	Inulin
A	1	32.4	436	129	28.9	351	89
	2	29.1	352	149	24.3	346	60
	3	34.3	383	133	26.7	356	56
C	1	19.3	508	70.4	29.4	637	107
	2	13.8	343	58.1	20.7	364	80
	3	12.9	428	64.2	27.6	282	81

Values are in ml/min corrected to standard surface area. Assays were by the dextran-coated charcoal method.

have demonstrated a progressive rise in the ratio of E_3-16G to E_3-3S-16G in amniotic fluid as normal pregnancy advances, whereas this ratio is abnormal in pregnancy complicated by Rh-isoimmunization. The renal clearances of estriol conjugates in normal term pregnant women show marked differences for different conjugates. Further study of the behavior of the different conjugates may be rewarding in understanding clinical complications of pregnancy.

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