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ESTRIOL ASSAYS IN OBSTETRICS

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SUMMARY

Estriol-6-(O-carboxymethyl) oxime (E_3-6-CMO) and estriol-4-azobenzoic acid (E_3-4-ABA) were coupled to BSA and compared with regard to antigenicity and antiserum specificity as well as suitability for radioimmunoassay (RIA) of unconjugated and total plasma estriol (E_3) and, if possible, of immunoreactive E_3 in unextracted plasma. Anti-E_3 titers were consistently higher in response to E_3-6-CMO-BSA than to E_3-4-ABA-BSA. Antisera against E_3-6-CMO-BSA cross-reacted up to 220% with 6-oxoestriol, but exhibited ring D specificity comparable to that observed with sera against E_3-4-ABA-BSA which cross-reacted with estriol-3-sulfate (E_3-3S) and estriol-3-glucosiduronate (E_3-3G) considerably more than E_3-6-CMO-BSA antisera.

RIAs for unconjugated and total E_3 in third trimester pregnancy plasma were established utilizing either type of antiserum for unconjugated E_3. The specificity of both types of antisera sufficed to render purification of E_3 extracts prior to RIA superfluous. Anti-E_3-4-ABA-BSA sera facilitated the RIA of immunoreactive E_3 in 10 μl of unextracted plasma measuring unconjugated E_3 plus E_3-3S and E_3-3G. All three assays could be performed with an inter-assay coefficient of variation of 10% or less. Normal values for unconjugated and total plasma E_3, measured in 300 women with uncomplicated pregnancies, averaged 6·9, 10·7 and 16 ng/ml and 98, 135 and 196 ng/ml at 30–32, 33–36 and 37–40 weeks of gestation, respectively. Unconjugated E_3 averaged 8·8% of total plasma E_3 concentrations. Immunoreactive plasma E_3 concentrations, on the average, were 6 times larger than unconjugated E_3 and amounted to about 50% of total plasma E_3 levels.

INTRODUCTION

For more than a decade urinary estriol (E_3) excretion served as an indicator of feto-placental function in high-risk pregnancies, despite inconvenience and inaccuracy inherent in 24-h urine collections, because plasma estriol assays could not be performed routinely by most clinical laboratories. The fluorometric plasma E_3 method of Nachtigall *et al.* [1] was the first and, except for some modifications [2, 3], the only clinically practical procedure to measure total (i.e., unconjugated and conjugated) plasma E_3 during the second half of gestation until radioligand assays became available [4–9]. These assays, although sufficiently sensitive to permit quantitation of unconjugated plasma E_3 [5–9], required solvent partition and/or chromatography because neither the uterine cytosol [4, 5] nor the antisera [6–9] used were sufficiently specific for E_3. Thus, in order to develop rapid and economic radioimmunoassays (RIA) for unconjugated or total plasma E_3, it was essential to obtain E_3-specific antisera. Two promising routes had been reported: protein conjugates of (1) estriol-6-(O-carboxymethyl) oxime (E_3-6-CMO) [10–14], and (2) estriol-4-azobenzoic acid (E_3-4-ABA) [15, 16], both of which are readily available and/or synthesized. The present study was undertaken to compare both antigens with regard to antigenicity and antiserum specificity as well as suitability for RIA of unconjugated and total plasma E_3, and if possible, of immunoreactive E_3 directly in plasma. RIA procedures for unconjugated, total and immunoreactive plasma E_3 and normal values obtained in some 300 women with uncomplicated pregnancies between 30 and 40 weeks of gestation will be presented. While this study was in progress, Den *et al.* [17] and Goebel and Kuss [18] reported RIA procedures for unconjugated E_3 in pregnancy plasma utilizing antisera against E_3-6-CMO-protein conjugates. A radioimmunoassay for unconjugated plasma E_3 utilizing either anti-E_3-6-CMO or anti-E_3-4-ABA sera has recently been reported from this laboratory [19].

EXPERIMENTAL

Abbreviations and trivial names

Dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; 16-epiestriol, 1,3,5(10)-estratriene-3,16β,17β-triol; estradiol-17β(E_2), 1,3,5(10)-estratriene-3,17β-diol; estradiol-3-sulfate (E_2-3S), 17β-hydroxy-1,3,5(10)-estratrien-3-yl-sulfate; estradiol-3-glucosiduronate (E_2-3G), 17β-hydroxy-1,3,5(10)-estratrien-3-yl-β-D-glucopyranosiduronate; estriol (E_3), 1,3,5(10)-estratriene-3,16α,17β-triol; estriol-3-sulfate (E_3-3S), 16α,17β-dihydroxy-1,3,5(10)-estratrien-3-yl-sulfate; estriol-3-glucosiduronate (E_3-3G), 16α,17β-dihydroxy-1,3,5(10)-estratrien-3-yl-β-D-glucopyranosiduronate; estriol-16-glucosiduronate (E_3-16G), 3,17β-dihydroxy-1,3,5(10)-estratrien-16α-yl-β-D-glucopyranosidur-

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onate; estriol-3-sulfate-16-glucosiduronate (E_3-3S,16G), 17β-hydroxy-1,3,5(10)-estratrien-3-yl-sulfate-16α-yl-β-D-glucopyranosiduronate; estriol-6-(O-carboxymethyl) oxime (E_3-6-CMO), 3,16α,17β-trihydroxy-1,3,5(10)-estratrien-6-one 6-(O-carboxymethyl) oxime; estrone sulfate (E_1-S), 17-oxo-1,3,5(10)-estratrien-3-yl-sulfate; estrone glucosiduronate (E_1-G), 17-oxo-1,3,5(10)-estratrien-3-yl-β-D-glucopyranosiduronate; 2-hydroxyestriol, 1,3,5(10)-estratriene-2,3,16α,17β-tetrol; 6-oxoestriol (6-oxo-E_3), 3,16α,17β-trihydroxy-1,3,5(10)-estratrien-6-one; progesterone, 4-pregnene-3,20-dione; testosterone, 17β-hydroxy-4-androsten-3-one.

Solvents, reagents and steroids

Solvents and reagents were of analytical grade unless otherwise stated. All solvents were distilled prior to use with the exception of diethyl ether (USP, Mallinckrodt, St. Louis, Mo., U.S.A.) which was used from cans opened on the day of use. [6,7-^{3}H]Estriol (S.A., 53 Ci/mmol) was purchased from New England Nuclear (Boston, Mass., U.S.A.), analyzed for radiochemical homogeneity by subjecting an aliquot to paper partition chromatography, and stored in ethanol:benzene (7:3/v:v) at 3°C. Non-labeled steroids were obtained from Steraloids, Inc. (Pawling, N.Y., U.S.A.). ^{14}C-labeled estriol-3-sulfate (E_3-3S), estriol-3-glucosiduronate (E_3-3G), estriol-16-glucosiduronate (E_3-16G) and estriol-3-sulfate-16-glucosiduronate (E_3-3S,16G) with low specific activity (29 mCi/mmol) were prepared biosynthetically and analyzed for radiochemical purity as previously reported [20]. ^{3}H-E_3-3S,16G with a S.A. of 5·8 Ci/mmol was biosynthesized according to the method of Levitz *et al.* [21].

Subjects

Antecubital venous blood was obtained from some 300 pregnant women between 30 and 40 weeks of gestation who attended our prenatal clinics and had uncomplicated and well-dated pregnancies. All blood specimens were collected into EDTA-containing vacutainers. The plasma was separated immediately by centrifugation and stored at −15°C until it was analyzed.

Generation and evaluation of antisera

E_3-6-CMO was purchased from Steraloids, Inc. (Pawling, N.Y., U.S.A.) and E_3-4-ABA was prepared according to the method of Gross *et al.* [15, 16]. Both E_3 derivatives were linked to bovine serum albumin (BSA) according to the mixed anhydride procedure described by Erlanger *et al.* [22] resulting in the incorporation of 24 moles of either E_3 derivative per mole of BSA as previously reported [19]. Twelve young male New Zealand rabbits were immunized, six against E_3-6-CMO-BSA and six against E_3-4-ABA-BSA, and bled as outlined in Figs. 1 and 2. Antiserum titers were determined by incubating 0·10 ml serum aliquots, serially diluted with phosphate-gelatin (PG) buffer, with 0·25 ml of PG buffer containing 2500 c.p.m. of ^{3}H-E_3 (30 pg) for 3 h after which bound and unbound ^{3}H-E_3 was separated by Dextran-coated charcoal as previously described [19]. The antiserum dilution at which 40% of the ^{3}H-E_3 added was bound to the antibody is listed as antiserum titer because this antibody concentration provided most suitable standard curves in this study. Thus, the titers reported indicate initial antiserum dilution so that 1 ml of an antiserum with a titer of 1:n will suffice for 10 n RIA tubes. Cross-reaction of various estrogens, E_3 conjugates, and neutral steroids with E_3 was determined for various antisera as outlined by Thorneycroft *et al.* [23]. Appropriate amounts of steroids were dissolved in 70% ethanol, serially diluted and, in 20 μl aliquots, dispensed into 10 × 75 mm RIA tubes to which 180 μl of PG buffer, 50 μl of PG buffer containing 2500 c.p.m. of ^{3}H-E_3 and 100 μl of appropriately diluted antiserum was added as previously described.

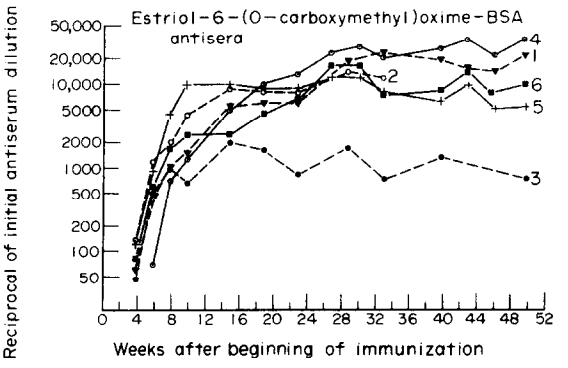

Fig. 1. Reciprocal of initial dilution at which 0·1 ml antiserum binds 40% of 2500 c.p.m. of tritiated estriol (30 pg) in a total incubation volume of 0·35 ml, representing anti-estriol titers in six rabbits immunized with estriol-6-(O-carboxymethyl) oxime coupled to bovine serum albumin. Rabbits number 1–3 received 200 μg of antigen at 0, 11 and 25 weeks while rabbits number 4–6 were immunized with 1 mg at 0, 1, 2, 3, 4 and 11 weeks.

Estriol radioimmunoassays

Radioimmunoassays were developed for (1) unconjugated and (2) total plasma E_3 as well as for (3) immunoassayable E_3 in unextracted plasma.

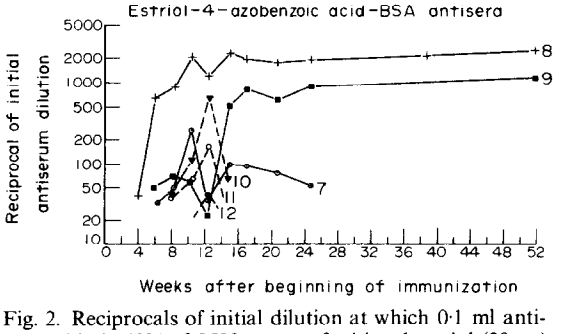

Fig. 2. Reciprocals of initial dilution at which 0·1 ml antiserum binds 40% of 2500 c.p.m. of tritiated estriol (30 pg) in a total incubation volume of 0·35 ml, representing anti-estriol titers in six rabbits immunized with estriol-4-azobenzoic acid coupled to BSA. Rabbits number 7–9 were immunized with 1 mg of antigen at 0, 1, 2, 3, 4 and 12 weeks while rabbits number 10–12 received 200 μg of antigen at 0 weeks, and 1 mg at 6 weeks.

1. Unconjugated E_3 was determined as recently described [19]. In brief, 500 c.p.m. of ^3H-E_3 were added as internal standard to 0·5 ml of plasma prior to extraction with 3 ml of 12% ethyl acetate in n-hexane, followed by a second extraction with 3 ml of 40% ethyl acetate in n-hexane. The latter extract was evaporated and redissolved in 0·4 ml of 70% ethanol, of which 0·20 ml was subjected to liquid scintillation counting and 20 μl, in triplicate, was taken for RIA using either E_3-6-CMO-BSA or E_3-4-ABA-BSA antiserum. Dextran-coated charcoal was used to separate bound and unbound ^3H-E_3. Logit transformation [24] was employed to construct standard curves and to calculate E_3 concentrations of plasma samples using a programmable Monroe model 1766 electronic calculator. Logit B/B_0 vs log dose standard curves [25] were linear between 7·8 pg and 2 ng of E_3.

2. Total plasma E_3 was determined in 0·1 ml plasma aliquots to which 600 c.p.m. of ^3H-E_3-3S,16G, dissolved in 0·1 ml of distilled water, was added as internal standard. Upon addition of 1·8 ml of diluted HCl (17 ml of concentrated HCl diluted with distilled water to 100 ml), plasma aliquots were subjected to 40 min of hot acid hydrolysis in 16 × 150 mm disposable test tubes topped with a glass marble, using a Temp-Blok module heater (Lab-Line Instruments, Melrose Park, Ill., U.S.A.) set at 110°C. The hydrolysate was cooled and extracted twice with 4 ml of diethyl ether. The ether extract was washed with 2 ml of bicarbonate buffer [26] and thereafter with 2 ml of distilled water, and evaporated to dryness under nitrogen. The residue was dissolved in 0·6 ml of 70% ethanol of which 0·40 ml was subjected to liquid scintillation counting while 20 μl, in triplicate, was taken for RIA using antisera against E_3-6-CMO-BSA.

3. Immunoreactive E_3 was assayed without extraction from plasma. Ten microliter plasma aliquots, in triplicate, were dispensed into 10 × 75 mm RIA tubes. Twenty μl of 70% ethanol, 180 μl of PG buffer, 50 μl of PG buffer containing 2500 c.p.m. of ^3H-E_3 and 100 μl of appropriately diluted anti-E_3-4-ABA-BSA serum were added to each RIA tube. Bound and unbound ^3H-E_3 was separated by Dextran-coated charcoal after 3 h of incubation at 4°C. Ten microliters of male plasma was added to all standard curve tubes to compensate for the 10 μl of plasma entering the RIA tubes of all samples. It was found that the addition of 10 μl of male plasma caused a minor but significant shift in the standard curve.

RESULTS

I. *Antiserum titers and specificity*

Within 15 weeks after immunization with E_3-6-CMO-BSA was begun, all six rabbits immunized responded with useful anti-E_3 titers ranging from 1:2000 to 1:10,000 initial dilution (Fig. 1). Persistently high antibody titers were achieved with antigen injections of 1 mg, each given at 0, 1, 2, 3, 4 and 11 weeks to three of the six rabbits as well as with as little as 200 μg antigen injected at 0, 11 and 25 weeks in the three other rabbits.

Such good antibody titers were not achieved in response to immunization with E_3-4-ABA-BSA (Fig. 2). Only one of the six rabbits immunized with this antigen produced useful and persistent anti-E_3 titers.

As previously reported [19], antisera against E_3-6-CMO-BSA (type 1) and E_3-4-ABA-BSA (type 2) exhibited excellent ring D specificity averaging only 2% cross-reaction with estradiol-17β (E_2) and less than 0·01% with estrone (E_1) and lacked detectable cross-reaction with progesterone, dehydroepiandrosterone and testosterone. Cross-reaction of type 1 and type 2 antisera with 2-hydroxyestriol was 1·5 and 2·9%, respectively, suggesting that the type 2 antigen was E_3-4-ABA-BSA rather than a mixture of E_3-4-ABA-BSA and E_3-2-ABA-BSA. While anti-E_3-4-ABA-BSA serum cross-reacted only 3·8% with 6-oxoestriol (6-oxo-E_3), type 1 antisera cross-reacted up to 220% with 6-oxo-E_3. No antiserum cross-reacted with E_3-16G or E_3-3S,16G, but type 1 and type 2 antisera cross-reacted 51 and 140% with E_3-3S and 26 and 170% with E_3-3G, respectively. The displacement curves for E_3-3S and E_3-3G paralleled that for E_3 over a wide range of steroid concentrations when anti-E_3-4-ABA-BSA serum was used, but not with type 1 antisera, indicating that type 2 antisera would be suitable for E_3-3S and E_3-3G RIA.

As shown in Table 1, antibody specificity remained largely unchanged between 15 and 33 weeks after the first immunization, but decreased thereafter.

Table 1. Percent cross-reaction of antisera obtained from six rabbits at 15 (A), 23 (B), 33 (C) and 48 (D) weeks after immunization with estriol-6-(O-carboxymethyl) oxime-bovine serum albumin was begun.

Number of rabbits		1	2	3	4	5	6	Mean
Estriol		100	100	100	100	100	100	100
Estradiol-17β	A	6.3	0.37	0.23	2.0	0.34	0.90	1.7
	B	6.6	1.2	1.1	2.1	0.29	1.3	2.1
	C	6.8	0.19	1.3	1.4	0.11	1.1	1.8
	D	11.	—	—	3.6	< 0.01	1.4	4.0
6-oxoestriol	A	91	216	72	200	169	76	137
	B	90	110	75	130	71	75	92
	C	58	68	48	132	88	77	79
	D	52	—	—	177	209	127	141
16-epiestriol	A	1.9	11	1.4	15.7	2.0	1.5	5.6
	B	3.3	6.2	3.4	11.4	2.0	2.3	4.8
	C	1.5	6.8	2.4	16.6	2.0	1.3	5.1
	D	2.4	—	—	36.2	2.1	6.6	12.
2-hydroxyestriol	B	0.52	6.2	0.83	2.3	0.38	0.56	1.8
	C	0.38	2.7	1.80	0.7	0.49	0.52	1.1
	D	1.3	—	—	6.3	2.2	1.8	2.9

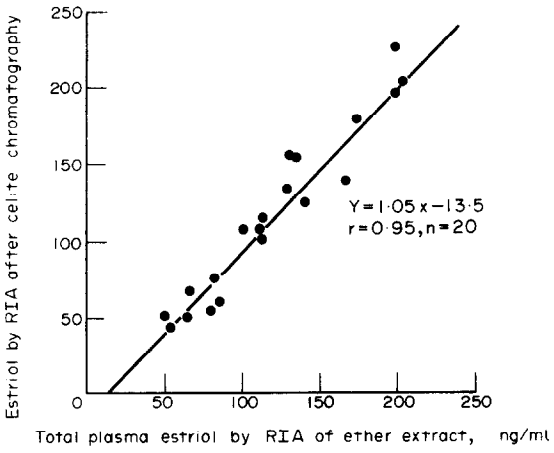

Fig. 3. Comparison of total plasma estriol concentrations determined by hot acid hydrolysis, ether extraction and radioimmunoassay with (y) and without (x) prior isolation of the estriol fraction by celite partition chromatography.

II. *Radioimmunoassays*

As previously reported [19], antisera against E_3-6-CMO-BSA and against E_3-4-ABA-BSA facilitated specific RIA procedures which did not require further purification of the plasma extract. Water and solvent blanks were essentially 0, the sensitivity was 1 ng/ml, intra- and inter-assay coefficients of variation averaged 10%, and the accuracy was maintained by employing ^{3}H-E_3 as internal standard.

Water and solvent blanks in the RIA for total plasma E_3 were virtually 0, and 5 ng/ml was measured with a coefficient of variation of 10%. The inter-assay coefficient of variation was 6·8%. The accuracy of the method was achieved through the use of ^{3}H-E_3-3S,16G as internal standard. The specificity of the procedure was examined by estimating total plasma E_3 with and without subjecting the ether extract to celite partition chromatography prior to RIA. As previously reported [19], this celite partition chromatography separates E_3 from 6-oxo-E_3, the most important cross-reacting compound, as well as from other estrogens. The regression analysis depicted in Fig. 3 demonstrates good agreement between the results obtained with and without celite partition chromatography, indicating that the RIA described is sufficiently specific to measure total plasma E_3 in late pregnancy plasma.

Immunoreactive E_3 in unextracted plasma was measured with an inter-assay coefficient of variation of 7·7%. As little as 3 ng/ml could be determined with a coefficient of variation of 10%. As the anti-E_3-4-ABA-BSA serum used in this direct RIA cross-reacted 140% with E_3-3S and 170% with E_3-3G, but not with E_3-16G nor with E_3-3S,16G, and as it exhibited excellent ring D specificity, it was assumed that this method would measure unconjugated E_3 plus E_3-3S and E_3-3G.

III. *Normal values during the third trimester*

Plasma concentrations of unconjugated, total and immunoreactive E_3 as measured in 300 women with well-dated and uncomplicated pregnancies between 30 and 40 weeks of gestation are depicted in Figs. 4–6. Statistical analysis revealed a larger spread of values above than below the medians calculated for each week of gestation for unconjugated, total and immunoreactive E_3. All means, medians and 95% confidence limits, as calculated by Rankit analysis [27, 28], are listed in Table 2.

When comparing unconjugated with total plasma E_3 in each of the 300 plasma samples, unconjugated E_3 averaged 8·8% of total plasma E_3. Immunoreactive plasma E_3 concentrations, on the average, were 5·9 times higher than unconjugated plasma E_3, and averaged 49% of total plasma E_3. Regression analysis of 300 data pairs revealed significant correlation

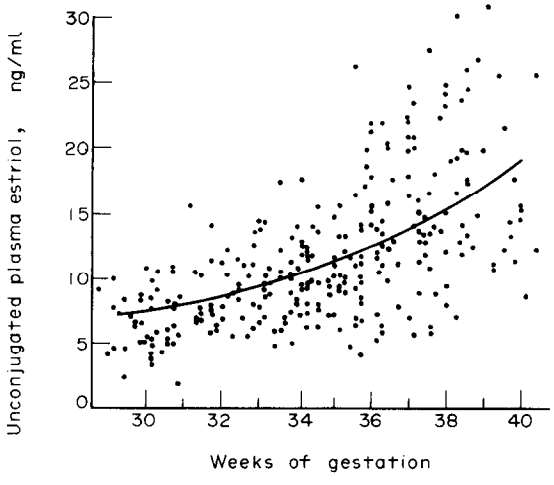

Fig. 4. Plasma concentrations of unconjugated estriol measured by radioimmunoassay of the plasma extract in 300 women with uncomplicated pregnancies. The ascending line represents averages calculated for each week of gestation.

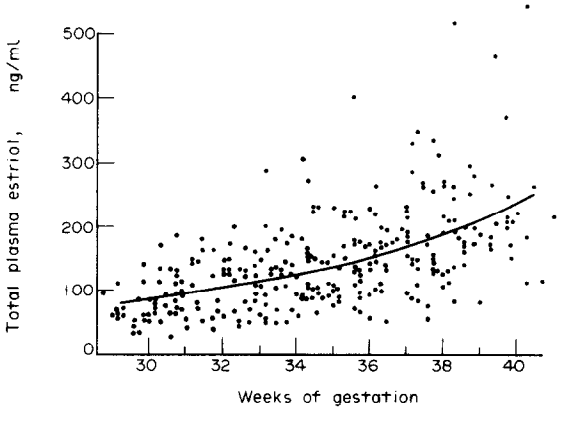

Fig. 5. Plasma concentrations of total estriol measured by radioimmunoassay following hydrolysis and ether extraction in 300 women with uncomplicated pregnancies. The ascending line represents averages calculated for each week of gestation.

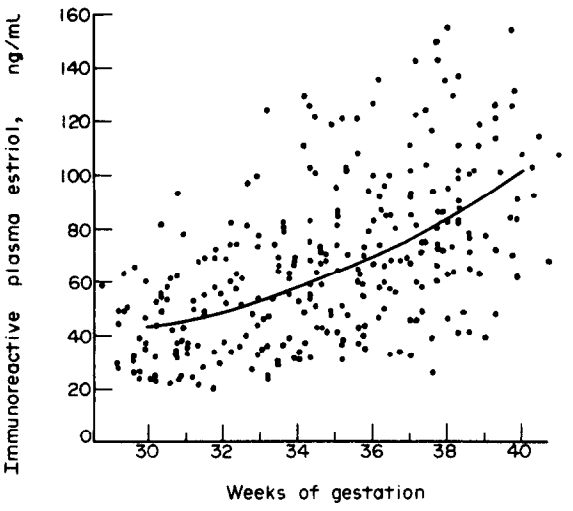

Fig. 6. Plasma concentrations of immunoreactive estriol comprising predominantly unconjugated estriol, estriol-3-sulfate and estriol-3-glucosiduronate, measured in 300 women with uncomplicated pregnancies by direct radioimmunoassay without plasma extraction. The ascending line represents averages calculated for each week of gestation.

between unconjugated and total ($r = 0{\cdot}813$), unconjugated and direct ($r = 0{\cdot}727$) as well as total and direct ($r = 0{\cdot}770$) plasma E_3.

DISCUSSION

The data presented indicate that both E_3-6-CMO-BSA and E_3-4-ABA-BSA generate antisera of high E_3-specificity, facilitating the development of RIA procedures for unconjugated and total plasma E_3 during the second half of gestation without need for purification of the plasma extract prior to RIA. The marked cross-reaction of anti-E_3-6-CMO-BSA sera with 6-oxo-E_3 appears irrelevant with regard to E_3 RIA as late pregnancy plasma was found to contain little 6-oxo-E_3 in relation to unconjugated E_3 [19]. Antisera against E_3-4-ABA-BSA exhibited considerable cross-reaction with E_3-3S and E_3-3G. This cross-reaction facilitates a direct RIA of E_3, E_3-3S and E_3-3G in unextracted plasma. The results obtained with this immunoreactive plasma E_3 RIA correlated with unconjugated and total plasma E_3. It remains to be established whether this direct and highly economical RIA will be of clinical value, perhaps as a screening procedure to identify abnormally low estriol production in a large out-patient population.

Our data indicate further that E_3-4-ABA-BSA antisera may be used for RIA of E_3-3S as well as E_3-3G. One may conclude that antisera against protein conjugates of estrone- and estradiol-4-azobenzoic acid would be equally suitable for the RIA of estrone-sulfate (E_1-S), estrone-glucosiduronate (E_1-G), estradiol-3-sulfate (E_2-3S) and estradiol-3-glucosiduronate (E_2-3G), respectively. This route of obtaining antisera for the RIA of E_1-S, E_1-G, E_2-3S and E_2-3G appears especially attractive as estrogen-4-azobenzoic acid-protein conjugates are more readily synthesized than protein conjugates of E_1-S, E_1-G, E_2-3S and E_2-3G.

While both types of antisera provided equally specific RIAs of unconjugated plasma E_3, antibody titers were markedly higher in response to E_3-6-CMO-BSA, suggesting that this antigen is preferable to E_3-4-ABA-BSA if the objective is to obtain consistently high titer antisera for the RIA of unconjugated and/or total plasma E_3. Antibody specificity varied between animals, but remained rather consistent in individual animals for up to 33 weeks after the beginning of immunization. Thereafter specificity began to decline. These indicate that there are about 20 weeks during which high titer antisera with relatively unchanged specificity may be sampled. This observation is in agreement with data reported by Walker *et al.* [13].

The results obtained with our RIA for unconjugated plasma E_3 agree with those reported by Den *et al.* [17] and by Goebel and Kuss [18] who used similar RIA methods utilizing antisera against E_3-6-CMO-BSA, but are larger than the normal values for unconjugated plasma E_3 in late pregnancy reported by Tulchinsky and Abraham [6] and by Cohen and Cohen [8] who employed celite partition chromatography prior to RIA with less specific antisera. Our mean total plasma E_3 concentrations determined for each week between 30 and 40 weeks of gestation are lower than those reported by Nachtigall *et al.* [1], but our lower 95% confidence limit, determined by Rankit analysis, [27, 28] agrees with the lower limit of normal values reported by these authors. As calcu-

Table 2. Means, medians and 95% confidence limits (by Rankit analysis) of unconjugated, total and immunoreactive plasma estriol (in ng per ml) determined in 300 women with uncomplicated pregnancies between 30 and 40 weeks of gestation.

Weeks of gestation	Number of assays	Unconjugated estriol			Total estriol			Immunoreactive estriol		
		Mean	Median	95% C.L.	Mean	Median	95% C.L.	Mean	Median	95% C.L.
30	25	7.2	6.2	3.2-12	74	67	31-140	37	35	20- 62
31	21	6.5	6.7	2.8-16	100	94	44-205	48	45	23- 83
32	21	7.1	8.2	5.0-14	123	105	35-330	54	48	19-120
33	27	9.1	9.3	4.6-20	125	108	37-300	60	53	22-125
34	29	10.2	9.6	3.7-23	126	109	45-260	61	54	22-135
35	39	10.4	11.8	6.1-17	138	128	62-260	62	56	29-110
36	33	12.9	13.0	5.0-27	148	140	48-350	70	61	22-166
37	30	14.6	13.3	5.6-31	172	157	74-330	75	70	33-150
38	36	15.5	14.0	5.5-33	197	184	59-570	89	83	35-196
39	14	17.0	16.3	9.1-29	214	194	132-280	94	82	48-160
40	25	17.7	16.5	7.3-37	230	205	95-460	98	102	50-170

lated from 300 data pairs, unconjugated E_3 comprised 8·8% of total plasma E_3. This percentage agrees closely with the 7·6% reported by Smith and Hagermann [29], but is smaller than that reported by us [20] using fluorometry rather than RIA and differs considerably from the percentage published by Touchstone *et al.* [30].

Each of the three plasma E_3 assays presented is easily established and performed in a clinical laboratory on a routine basis. However, it remains to be established which assay, if any, provides the clinically most useful answers, i.e., shows the steepest decrease in response to fetal distress and least diurnal and/or episodic fluctuations of the compound(s) it measures. Studies of E_3 transfer [31] and metabolism [32] in pregnant women suggest that unconjugated plasma E_3 should reflect feto-placental E_3 production most appropriately as its concentration depends largely upon feto-placental secretion and maternal hepatic clearance (conjugation) of E_3, whereas total plasma E_3, in addition, depends upon renal clearance of various E_3 conjugates. Tulchinsky *et al.* [5] ruled out substantial diurnal variations of unconjugated plasma E_3 in late pregnancy. On the other hand, Selinger and Levitz [33] found that total plasma E_3 in late pregnancy displayed diurnal variations characterized by lower concentrations at 16:30 h than 8:00 or 21.00 h when measured on 2 consecutive days. Townsley *et al.* [34] also reported diurnal variations of total E_3, but to a lesser extent than Selinger and Levitz [33]. However, most recently Levitz *et al.* [35] presented evidence contradicting significant periodocity of plasma total E_3 concentrations, but indicating rather large episodic fluctuations. Ongoing studies in our laboratory appear to indicate about 10% lower unconjugated plasma E_3 concentrations at 9:00 and 12:00 than at 6:00, 16:00, 19:00 and 24:00 h and somewhat lower total plasma E_3 concentrations between 16:00 and 24:00 h than at 6:00, 9:00 and 12:00 h. According to these preliminary findings, minor diurnal variations of unconjugated and total plasma E_3 may exist but appear to be overshadowed by episodic fluctuations as reported by Levitz *et al.* [35] for total plasma E_3 suggesting that blood sampling at the same time every day may not eliminate considerable day to day fluctuations of unconjugated and/or total plasma E_3. Only a large-scale comparative clinical study with daily determinations of unconjugated and total plasma E_3 in high-risk pregnancies will permit to conclude which assay will be more valuable in obstetrics.

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