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SERUM LEVELS OF UNCONJUGATED ESTETROL-(1,3,5(10)-ESTRATRIEN 1,15α,16α,17β-TETROL) DURING HUMAN PREGNANCY

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SUMMARY

A radioimmunoassay is described for the measurement of unconjugated estriol (E_3) and estetrol (E_4) in pregnancy serum. Specimens were obtained from about 70 women at 20–42 weeks of gestation. During the last half of pregnancy the concentration of E_4 was found to increase. The mean serum concentration of unconjugated E_4 during the 40th week was 2·2 ng/ml. The E_3/E_4 ratio was about 8 and remained nearly constant throughout pregnancy, although the increase of E_4 was relatively greater than the increase of E_3 during the last few weeks of gestation.

ESTETROL* (E_4), isolated from urine of newborns [1] and pregnant women [2–4], is a characteristic product of 15α hydroxylation of steroids in the fetus. Early experiments showed that isotopically labeled estradiol (E_2) injected into the amniotic fluid or directly into the foetus was extensively converted to E_4 while ^{14}C-labeled E_2 injected simultaneously into a maternal vein was not [3]. Similar observations were made with labeled estriol (E_3) [5]. Subsequently, Younglai and Solomon [6] demonstrated the conversion of C_{19} steroids to E_4. These experiments indicate that both estrogens and C_{19} steroids are precursors of E_4, although the relative contribution of each is not known.

The concentration of E_4 in blood, urine, and amniotic fluid is being evaluated, particularly in relation to E_3, as an index of the condition of the foetus. Recent studies have utilized gas–liquid chromatography to analyze the urinary excretion of E_4 in normal [7] and abnormal [8] pregnancies.

This article describes the measurement of concentrations of unconjugated E_3 and E_4 in serum during normal pregnancy. The estrogens were measured by radioimmunoassay using a sheep antiserum obtained with an antigen prepared by conjugating estriol 16, 17-dihemisuccinate to bovine serum albumin. As shown is a previous publication where the method is described [9], E_3 and E_4 cross-react with this antiserum.

EXPERIMENTAL

Materials

Tracers. 6,7-^{3}H-estriol (s.a. = 50 Ci/mmole), purchased from New England Nuclear Corporation was purified by column chromatography on Celite using the solvent system isooctane–chloroform–methanol–water, 8 : 12 : 7 : 3 (by vol.). The

*Abbreviations: E_2, estradiol-17β; E_3, estriol; E_4, estetrol; 1,3,5(10)-estratrien-3,15α,16α,17β-tetrol.

purified tracer appeared radiochemically homogeneous when rechromatographed on Sephadex LH-20.

4-^{14}C-estriol (s.a. = 53·4 mCi/mmole), purchased from Amersham Searle Co., was purified by column chromatography as described for [^{3}H]-E_3.

Randomly labeled [^{3}H]-estetrol (s.a. about 4·0 Ci/mmole) was a gift of Dr. Jack Fishman, Institute of Steroid Research, Montefiore Hospital, N.Y. This material was repurified by chromatography on Sephadex LH-20.

[6.7-^{3}H]-estradiol (s.a. = 40 Ci/mmole), purchased from New England Nuclear Company, was purified by paper chromatography using the solvent system benzene–heptane–methanol–water, 7 : 3 : 8 : 2 (by vol.).

Unlabeled steroids. Crystalline estetrol was a gift of Dr. S. Solomon, McGill University, Montreal.

Estriol was purchased from Mann-Schwartz Laboratory, N.Y.

Solvents. Ethyl acetate (Mallinkrodt Company, Analytical Reagent grade), redistilled over potassium carbonate, was used within a week.

Benzene, methanol (Fisher, spectroanalyzed grade) and toluene (Mallinkrodt, Analytical Reagent grade) did not require further purification.

Dioxane (Fisher Company) was refluxed for 4 h over sodium hydroxide pellets and distilled.

A 0·01 M phosphate buffer solution, pH 7·4, was made in 0·88% sodium chloride.

Sephadex LH-20. Sephadex LH-20 was purchased from Pharmacia Company N.Y. This material was soaked in a mixture of methanol and benzene (1 : 4) for 4 days before use.

METHODS

A known amount of tritiated E_3 and E_4 was added to an aliquot of each serum sample and the estrogens were then extracted with ethyl acetate. Following chromatography of the extract on a Sephadex LH-20 column, the concentration of estrogens was determined by radioimmunoassay as described elsewhere [9]. Methanol was added to another portion of the serum sample and an aliquot of the methanolic supernatant was used to measure "total estrogens" [9].

Measurement of radioactivity

All measurements of radioactivity were performed with a liquid scintillation spectrometer (Beckman LS-200). Two types of scintillation fluids were used. One of them consisted of a solution of 0·4% POP (2,5-diphenyloxazole) and 0·05% POPOP (1,4-bis 2-(5-phenyloxazolyl)benzene) in toluene. The other consisted of a solution of the same phosphors (0·4 and 0·03%, respectively) in dioxane containing 100 g of naphthalene per l. The efficiencies of counting ^{3}H in toluene and dioxane solutions were, respectively, 46 and 31%.

Indicators

A methanolic solution of approximately 13,000 dpm of [^{3}H]-E_3 (0·03 ng) and 9700 dpm of [^{3}H]-E_4 (0·2 ng) was evaporated to dryness in a tube into which the sample of serum (5 or 10 ml, depending upon gestational age) was then added. The amounts of indicator proved sufficient to estimate accurately losses without interfering with the radioimmunoassay. Corrections for weight added by the indicators were made when necessary.

Extraction

The efficiency of different solvents in extracting E_3 and E_4 from serum was tested. It was found that when serum was extracted once with double volume and twice with an equal volume of ethyl acetate, 83% of the E_4 was removed. Ether was less effective than ethyl acetate for the extraction of E_4.

Chromatography

A 1 × 25 cm all glass column containing 3 g of Sephadex LH-20 was used according to the directions of Mikhail *et al.* [10, 11]. The solvent system benzene–methanol, 4 v/v–1:, was found adequate to separate 3 distinct peaks of E_2, E_3 and E_4, as shown in Fig. 1. The flow rate was 0·4 ml per min and 1·0 ml fractions were collected; 1/50 aliquots of the fractions expected to contain E_3 and E_4 were counted to confirm the position of the peaks of radioactivity for each sample. Two fractions containing most of the E_3 (e.g. Nos. 19 and 20 in Fig. 1) were analyzed separately. The peak represented about 25% of the indicator added. The contents of the tube with most of the labeled E_4 and the subsequent tube (e.g. Nos. 25 and 26 in Fig. 1), which together contained about 40% of the indicator, were combined. A 20% aliquot of the combined fractions of E_4 and 40% of each of the E_3 fractions were taken for accurate determination of recoveries. Estetrol was counted in the dioxane phosphor solution and E_3 in the toluene phosphor solution.

Radioimmunoassay

Measurement of concentrations of E_3 *and* E_4. The radioimmunoassay was performed as described previously [9]. In brief, this assay involved the following

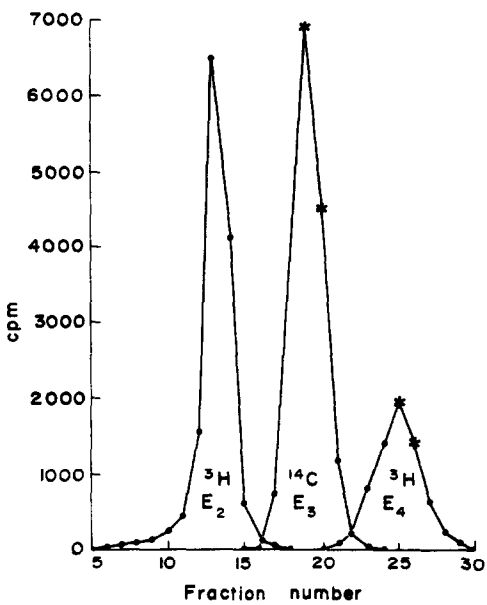

Fig. 1. Chromatogram of a mixture of [^{3}H]–E_2, [^{14}C]–E_3 and [^{3}H]–E_4 passed through a column of 3 g of Sephadex LH-20. Solvent System: benzene–methanol, 4–1; flow rate: 0·4 ml/min; fraction size: 1 ml. The asterisks indicate the fractions subjected to radioimmunoassay to measure E_3 and E_4 levels in pregnancy serum.

steps:

– Addition of known amounts of E_3 and E_4 ranging from 0 to 6 ng to tubes containing appropriate amounts of dried reagent blank (solvent passed through the LH-20 column). Six tubes, in duplicate, were prepared for each of the compounds to determine the reference curves. Samples (2 aliquots of each fraction of E_3, 1 of E_4) were transferred to tubes and the solvents were evaporated under nitrogen.

– Addition of antiserum and ^3H–E_3 (approximately 65,000 dpm buffer to all tubes. The antiserum contained male plasma to facilitate separation of proteins.

– Equilibration in an ice-bath and addition of ammonium sulphate; centrifugation at 8,000 *g* (Sorvall RB-2 centrifuge) to separate precipitate proteins.

– Counting of 0·5 ml aliquots of the supernatant after vigorous mixing with 10 ml of the toluene phosphor solution. The radioactivity measured was expressed as percentage of added ^3H–E_3 left unbound. The amount of labeled material introduced with the sample was included in the estimation of the fraction of unbound radioactivity.

Estimation of total estrogens

This determination has been described previously[9]. As suggested in that article appropriate amounts of dried methanolic extract of male plasma were included in each tube used to determine the standard curve for this assay.

Computation

Concentrations of estrogens in serum were calculated from the experimental data using a desk computer (Olivetti, Programma 101). Correlations of these results were performed with the aid of a digital computer (CDC 3300). Plots and statistical parameters for these correlations were obtained.

RESULTS

Separation of E_3 *and* E_4

Since there is more E_3 than E_4 in plasma and since E_3 competes more efficiently than E_4 with the labeled E_3 for the antibody, it was important to ascertain that no E_3 was contaminating the E_4 fraction after chromatography on LH-20. Even though the separation of labeled E_3 from E_4 appeared to be complete (Fig. 1), a series of experiments were conducted in which E_3 was added to male serum and measured in the E_4 region of the chromagram. The amount of E_3 used, 20·0 ng per ml of serum, represents the highest concentration found in pregnancy serum. Referring to Fig. 1, the concentration of E_3 in serum calculated from measurements in fraction 19 (E_3 peak) was 19·3 ng/ml. Assay of the combined fractions 23 and 24 (early fractions of the E_4 peak) erroneously indicated the presence of 1·2 ng of E_4 per ml of serum. This value is due to tailing of E_3 during chromatography. However, the value of E_4 which would be estimated from measurements of the combined fractions 25 and 26 (routinely used in the E_4 assay) was only 0·2 ng of E_4 per ml of serum. This value is slightly higher than the male serum blank, which corresponded to 0·1 ng E_4/ml serum. It can be concluded that the separation of E_3 and E_4 is complete when fractions 25 and 26 (Fig. 1) are used for the assay of E_4.

Detection of E_3 *and* E_4 *added to male serum*

Known amounts of E_3 and E_4 were added to male serum and the assays for these compounds were performed. The results are shown in Figs. 2 and 3. Each point in these curves represents the average of 4 to 8 determinations.

Serum concentrations of unconjugated E_3, E_4 *and "total estrogens"*

The results obtained with pregnancy sera are shown in graphical form, using reproductions of the computer print-outs. Figures 4 and 5 represent the plots of the concentrations of E_3 and E_4 versus the gestational age. The serum unconjugated E_3 curve shows the typical increase with time of gestation as well as the characteristic wide dispersion of values observed in urinary E_3. A similar pattern is noted for E_4. In Fig. 6, the ratios of the concentrations of E_3 and E_4 plotted vs. gestational age are shown. This ratio is about 8 and remains approximately constant during the development of the foetus. A lowering of the ratio

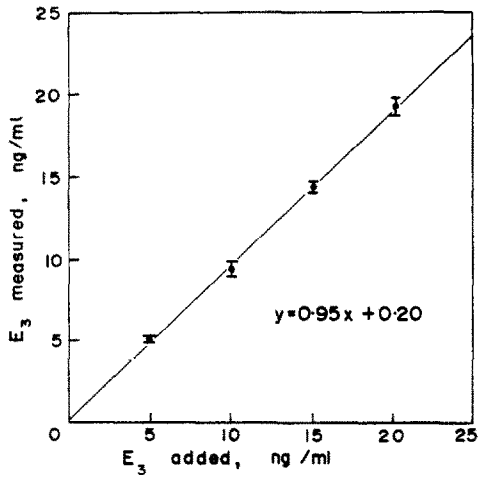

Fig. 2.

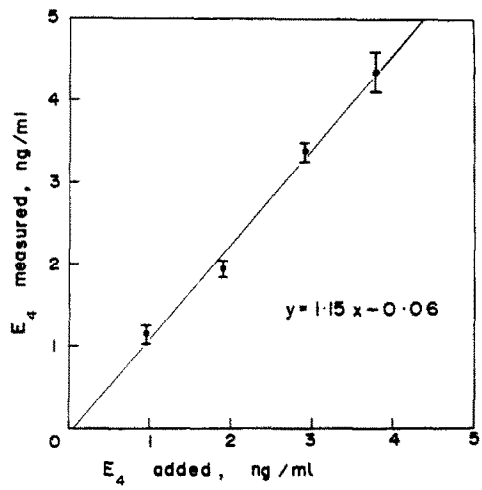

Fig. 3.

Figs. 2 and 3. Amounts of E_3 and E_4 measured in human male serum after addition of known amounts of these compounds (mean ± SE). Regression lines calculated by the least squares method. Each point is the average of 4–8 determinations.

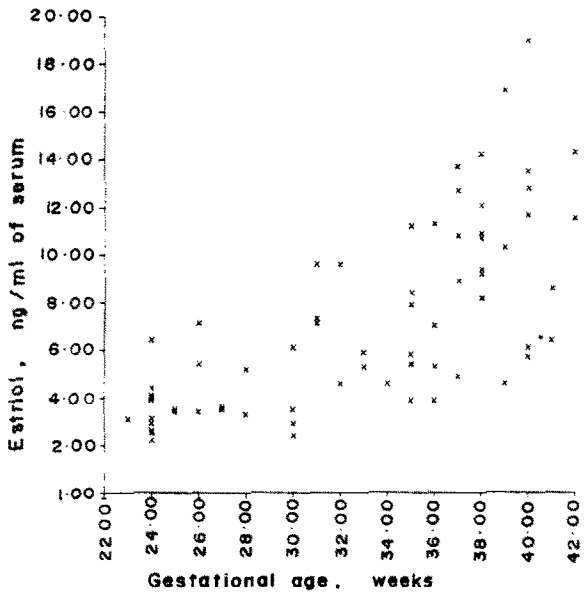

Fig. 4.

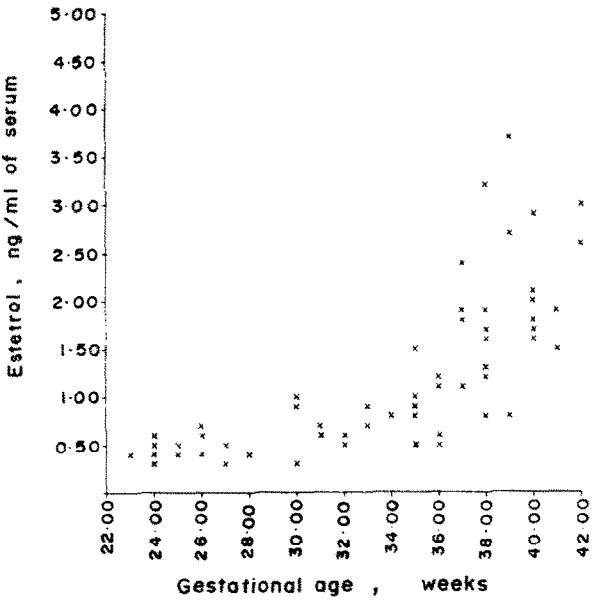

Fig. 5.

Figs. 4 and 5. Concentrations of E_3 and E_4 in serum vs. gestational age.

appears to occur at the end of gestation. Such an increase in the relative concentration of E_4 with respect to E_3 in serum is in agreement with the urinary excretion rates of these compounds [7].

Figure 7 shows the ratio of the concentrations of the "total estrogens", as determined by radioimmunoassay, to the concentration of unconjugated E_3 in serum as a function of gestational age. It is apparent from the graph that the

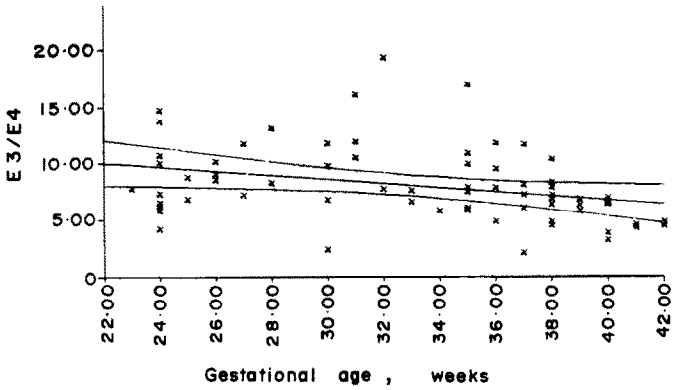

Fig. 6. Ratios of concentrations of E_3 and E_4 in serum versus gestational age. The upper and lower curves indicate 95% confidence limits, i.e. the probability that the true regression line lies within the region limited by these curves is 0·95.

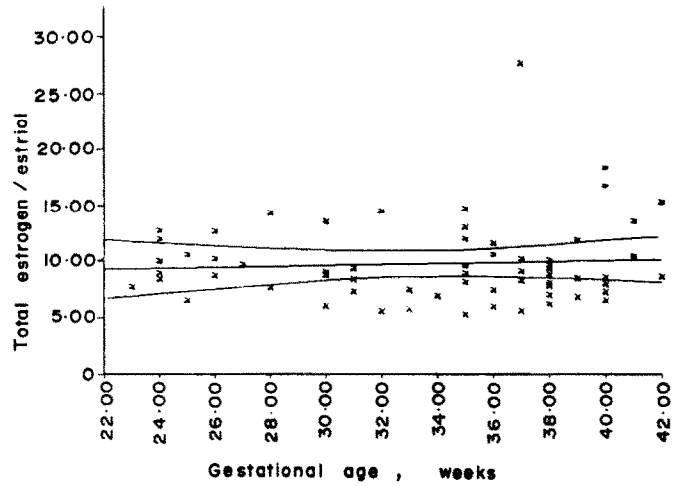

Fig. 7. Ratios of concentrations of "total estrogens" (see Ref. 9) to E_3 in serum versus gestational age. Outer lines indicate 95% confidence limits.

concentration of "total estrogens" is proportional to the concentration of unconjugated serum E_3.

The relative simplicity of the radioimmunoassay of E_3 and E_4 in serum makes this method suitable for the study of large numbers of samples. This procedure, which may also be applicable to the measurement of concentrations of unconjugated E_3 and E_4 in amniotic fluid, can be used to study the usefulness of E_4 as an indicator of the condition of the foetus.

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