Free and Conjugated Estrogens in Blood Plasma during Human Pregnancy*

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ABSTRACT: The concentrations of the free, glucosiduronate, and sulfate forms of estrone (E_1), estradiol-17β (E_2), and estriol (E_3) in maternal and umbilical cord blood are reported. Phenolsulfatase and β-glucuronidase hydrolyses followed extraction of the free ether-extractible estrogen. There was more E_1 and E_2 in the conjugated forms in the maternal than in cord plasma. E_1 sulfate (4.1 μg) was the major estrogen other than E_3 sulfate (4.4 μg 100 ml) found in the maternal blood. Both free E_1 and E_2 were found in higher concentrations that their glucosiduronates. Results for concentrations of the E_3 forms agreed with the previous work (J. C. Touchstone, J. W. Greene, Jr., R. C. McElroy, and T. Murawec [1963], *Biochemistry 2*, 653). No estrogen was found in the red cells by the present method.

Several studies describing estrogen levels in blood have been published recently (Diczfalusy and Magnusson, 1958; Roy and Mackay, 1962; Maner *et al.*, 1963; Aldercreutz, 1964). These reports have evaluated the total amounts of estrone, estradiol-17β, and estriol rather than differentiating between free and conjugated steroids. Recent reports from this laboratory describe a comparison of the conjugation of estriol in umbilical cord and in the maternal peripheral blood plasma (Touchstone *et al.*, 1963). A preliminary report described the conjugation of estrone and estradiol-17β in both cord and maternal blood (Touchstone and Murawec, 1963).

The present report gives details of methodology and results of quantitation of estrone, estradiol-17β, and estriol in plasma in the free form and after liberation by hydrolysis with β-glucuronidase and phenolsulfatase (Mylase P) in a larger series. Conjugates of estrone and estradiol-17β were found in larger quantity in the maternal blood than in cord blood. No estrogens were found in the red cells.

Apparatus and Column Conditions

The gas chromatograph used in this work was (Glowall Corp. Model A-110) equipped with a Lovelock detector using radium foil. The column, a glass coil (183 cm × 4 mm), was packed with a combination of 10% QF-1 (fluorosilicone polymer, Dow Corning) and 5% L-45 (methylsilicone polymer, Dow Corning) on Gas-Chrom Z, 80–100 mesh (Applied Science). The substrates were coated on the support in one application by dissolving them in methylene chloride (5–6 volumes × the weight of support) and evaporating the solvent with constant stirring after addition of the support. The packing was dried in a vacuum oven and packed in the glass coil under a vacuum. The columns were conditioned at 270° for 2 days with argon flow at an inlet pressure of 10 psi. Operating conditions were: column and detector temperature, 250°; flash temperature, 270°; inlet pressure, 30 psi; and voltage, 1000. Samples were dissolved in 25 μl of *t*-butyl alcohol and 2 to 4-μl aliquots were injected.

Preparation of Red Cells and Blood Plasma Extracts. Heparinized blood was collected from the antecubital vein of women in the third trimester of pregnancy. Blood was milked from the umbilical cord at the time of separation from the fetus and pooled. Pools of 100 ml or more were used to stay within limits of sensitivity of the method. After centrifugation the plasma was extracted within 30 minutes after removal or frozen until extraction was undertaken.

Plasma proteins were precipitated by addition of 4 volumes of acetone and removed by centrifugation. The acetone was evaporated *in vacuo* in a warm-water bath. The aqueous residue was then hydrolyzed and extracted in four steps, as follows:

(1) FREE ESTROGENS. The aqueous residue was extracted three times with equal volumes of ether and separated.

(2) GLUCOSIDURONATES. For each 100 ml of aqueous residue, 5 ml of 0.2 M monosodium maleate was added and the *p*H was adjusted to 6.8 with 0.2 M sodium hydroxide using a *p*H meter. Bacterial glucuronidase (Sigma; 1000 units/ml) was added and incubation was carried out at 37° for 24 hours. The freed steroids were extracted by partition three times with equal volumes of ether.

(3) SULFATES. The aqueous portion was adjusted to *p*H 6.0 by addition of 0.2 M maleic acid. Mylase P (Nutritional Biochemicals) was added (10 mg/ml)

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and incubation was carried out at 50° for 24 hours. Freed steroids were extracted as above.

(4) OTHER. The aqueous phase after enzyme hydrolysis was subjected to acid hydrolysis with HCl (15 volumes %) and the hydrolysates also were extracted with ether following the prescribed method.

The red blood cells were washed twice by suspension in isotonic saline and centrifugation. The cells were then hemolyzed by addition of (0.1 volume) water followed by precipitation with acetone. The aqueous residues were sequentially hydrolyzed and extracted as described for plasma.

The four ether phases were washed twice with 0.1 volume of 5% sodium bicarbonate and once with 0.1 volume of water and evaporated. The residue was dissolved in 30 ml of toluene and extracted three times with 20 ml of 1 N NaOH. The alkaline solution was back extracted with toluene. The combined alkali phases were neutralized to *p*H 6–7 with 5 N HCl. The phenols were separated by extraction three times with equal volumes of ether. Combined ether extracts were then washed with bicarbonate and water as above. The ether was evaporated and the residue was subjected to alumina column chromatography.

Alumina Column Chromatography. Activated alumina (Harshaw, catalyst grade) was washed with absolute ethanol and dried in a vacuum oven at 120° for 18 hours. It should be protected from moisture. One g of alumina was poured into a 5-ml syringe fitted with a 22-gauge needle and filled with benzene (Eberlein *et al.*, 1958). The extract was added to the column in benzene followed by benzene, 30 ml; 1% ethanol in benzene, 20 ml; 10% ethanol in benzene, 20 ml; and finally 20 ml of 50% ethanol in benzene. The fractions were evaporated in a vacuum oven and transferred with acetone into 1.5-ml test tubes and evaporated in a vacuum oven. *t*-Butyl alcohol, 25 μl, was added to each tube and aliquots were taken for gas chromatography or other identification procedures. Estrone and estradiol-17β are found in the 10% ethanol in benzene fraction while estriol is found in the 50% ethanol in benzene fraction.

Quantitation. The fractions from the alumina columns were subjected to gas chromatography and amounts were calculated by interpolation of the areas of the peaks for the individual steroids. The Bachman reagent (1939) was also used as a supplementary quantitative method for estriol. Quantitation with the Kober reagent was used for confirmation of estrone and estradiol-17β.

Precision of Method. The method of Snedecor (1952) was used to give an estimate of the precision of the determinations. In eight duplicate determinations of estrogen liberated by glucuronidase the standard deviation (SD) was 0.10 μg/100 ml for estrone and 0.25 μg/100 ml for estradiol-17β. For sulfatase hydrolysis the SD was 0.92 for estrone and 0.5 μg/100 ml for estradiol-17β. The SD for estriol determinations was 5.5 μg/100 ml after sulfate hydrolysis of conjugates in cord plasma.

Identity of the estrogens was verified by paper and thin-layer chromatography. The comparison of the

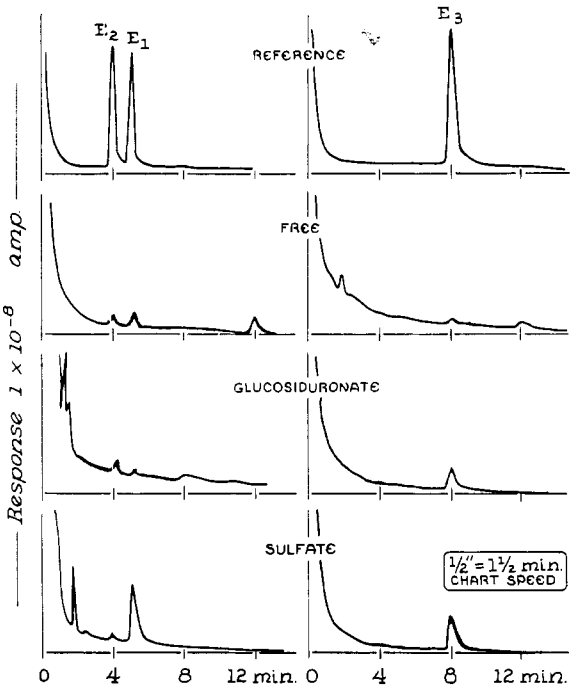

FIGURE 1: Gas chromatography of estrogens in maternal blood plasma. Left panels: 10% methanol in benzene fraction of the alumina column. Right panels: 50% methanol in benzene. Estrone (E_1), 1.0 μg; estradiol-17β (E_2), 1.0 μg; and estriol (E_3), 2.0 μg.

amounts seen on the chromatograms after color development with the ferric chloride–potassium ferricyanide reagent agreed well with the result of quantitation by gas chromatography. The values obtained for estriol concentration obtained by use of the Bachman color reaction and for estrone and estradiol-17β using the Kober reagent were confirmatory.

The column used for gas chromatography of estrogens in this work contained a mixed-phase packing. We have previously shown how the use of this type of packing is more reliable for separation of biological extracts since the effect obtained is that of separation on two different gas-chromatographic columns (Touchstone *et al.*, 1964). The identity of the conjugate fractions depends largely on the specificity of the enzymes used.

Results

Figure 1 shows the results of gas chromatography of pure estrone, estradiol-17β, and estriol after separation on the alumina column. Recoveries of these steroids from the column have been quantitative. The separation of estrogen fractions of an extract of maternal blood plasma by gas chromatogrophy is shown in Figure 1.

The recovery of estriol added to plasma averaged 80% in six determinations as reported in the previous paper (Touchstone *et al.*, 1963). Recoveries of estrone,

TABLE I: Concentrations of Estrone, Estradiol-17β, and Estriol (μg/100 ml).

	Free	Glucosid-uronate[a]	Sulfate[a,b]
	Maternal (9)[c]		
Estrone	1.42	0.44	4.1
	±0.96[d]	±0.16	±0.72
Estradiol-17β	0.80	0.69	1.57
	±0.27	±0.21	±0.69
Estriol	0.36	2.88	4.4
	±0.17	±0.75	±0.79
	Cord (8)[c]		
Estrone	1.3	0.35	0.84
	±0.26	±0.15	±0.14
Estradiol-17β	0.60	0.65	0.80
	0.10	±0.30	0.22
Estriol	6.9	20.0	94.1
	±1.29	±3.85	±12.03

[a] Measured as free estrogen after enzymatic hydrolysis. [b] Values based on initial hydrolysis since sequential hydrolysis will give false high values owing to presence of double conjugates. [c] (N) = number of determinations. [d] Mean ± standard error.

estradiol-17β, and estriol by the present method averaged 72% for three determinations.

The concentrations of estrone, estradiol-17β, and estriol are given in Table I. Estriol sulfate was the major estrogen found in both the maternal and cord plasma. This substance was found in concentrations as high as 170 μg/100 ml in cord plasma. Estrone and estradiol-17β were present in the highest concentrations in the maternal plasma as the sulfate although both were present also in the free form. Their concentrations were higher in the maternal than in the cord blood plasma. For all three estrogens the glucosiduronate was present in lower concentration than the sulfate. Next to estriol sulfate, estrone sulfate was present in the highest concentration in the maternal blood. No estrogens were found in the red blood cells.

Discussion

The total amounts of the estrogens measured in this study agree with the results reported by other investigators. Estrone and estradiol-17β were also found in greater concentrations in maternal than in cord blood both by Roy and Mackay (1962) and by Maner *et al.* (1963). Several investigators including ourselves have indicated the high concentrations of estriol in cord blood plasma. The finding of no estrogens in the red cells is in agreement with the results of Maner *et al.* (1963).

Figures for free estrogens in plasma during late pregnancy are few and vary a great deal. There is some evidence from this and other work that there is a considerable proportion of free estradiol-17β. The values for estrone sulfate agree well with the reports of Purdy *et al.* (1961) (5 μg/100 ml plasma) and Aldercreutz (1964) (4.80 μg/100 ml). Comparable values for cord blood are not available for comparison.

The results for estriol do not reflect the double conjugation previously reported [Touchstone *et al.* (1963)]. Sequential hydrolysis was performed in four of the cases listed and the concentration for the 3-sulfo-16- and/or -17-glucosiduronate diconjugate was the same as those previously found. No evidence for double conjugation of estradiol-17β was found.

Estrogen sulfates appear to be important intermediates in the metabolism of the estrogens. The sulfates, particularly estrone sulfate, may be biologically active and of great physiological importance as previously pointed out by Aldercreutz (1962) and Purdy *et al.* (1961). Sulfurylation appears to be an important step in the metabolism of steroids since Drayer *et al.* (1964) showed that cholesterol can be transformed to its sulfate by the adrenal. Siiteri and MacDonald (1963) showed that dehydroisoandrosterone sulfate can be metabolized to the estrogens. However, until further studies on isolation of the conjugated estrogens in blood have been completed no definite conclusions about the true role of the estrogen sulfates can be made.

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