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URINARY EXCRETION OF 5β-PREGNANE-3α,6α,20α-TRIOL IN HUMAN GESTATION

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SUMMARY

The 5β-pregnane-3α,6α,20α-triol and 5β-pregnane-3α,6α,20β-triol obtained by reduction of 3α,6α-dihydroxy-5β-pregnan-20-one were converted to trimethylsilyl ether derivatives and analysed by gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC–MS).

After extraction, solvolysis and enzymatic hydrolysis of urinary steroid conjugates (from 19 normal pregnant women), the liberated steroids were separated by liquid-gel chromatography and were analysed by GLC and GLC–MS. The 5β-pregnane-3α,6α,20α-triol isolated and identified in the Sephadex LH-20 fractions 8 and 9, was present in urine from 15 pregnant women after the 16th week of gestation. After this time, this metabolite was found in a quantity between 0.20 to 2.90 mg/24 h, with a significant increase between the 26th to 30th week of the gestation.

With the present *in vivo* data, it is not possible to establish the exact enzymatic pathway involved in the biosynthesis of the 5β-pregnane-3α,6α,20α-triol. However, it is probable that the immediate precursor of this compound was the 3α,6α-dihydroxy-5β-pregnan-20-one, and that urinary excretion of 5β-pregnane-3α,6α,20α-triol reflected one part of hepatomaternal metabolism of 6-hydroxy-progesterone formed in the foeto-placental unit.

INTRODUCTION

In human gestation, it is known that the principal pathway for the elimination of steroid metabolites is urinary excretion. The major metabolites of progesterone found in the urine of pregnant women are 5β-pregnane-3α,20α-diol, 3α,16α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3α,20α-diol and 5β-pregnane-3α,17,20α-triol. In addition, previous *in vivo* investigations have shown that the conversion of progesterone to 6-oxy-21-deoxysteroids also seems to be an important metabolic pathway[1]. Some of these 6-oxygenated metabolites of progesterone were identified[2–5] and quantitated[6–8] in the urine of pregnant women. They are: 3α,6α-dihydroxy-5α-pregnan-20-one, 3α,6α-dihydroxy-5β-pregnan-20-one, 3β,6α-dihydroxy-5α-pregnan-20-one and 5α-pregnane-3α,6α,20α-triol. In this paper, the isolation, identification and quantitation of urinary 5β-pregnane-3α,6α,20α-triol found in pregnant women during the second and third trimester of human gestation is described.

EXPERIMENTAL

1. *Subjects.* Twenty-four-hour urine samples were collected from 19 normal pregnant women. Two samples were obtained from two different twin pregnancies. All patients presented a normal pregnancy and were delivered of mature live-born babies.

2. *Chemicals.* The solvents (reagent analytical grade), sodium borohydride, anhydrous sodium sulfate were purchased from Merck (Darmstadt, Germany). The dipyridinium sulfate (1 N) for extraction of steroid conjugates[9] was prepared by mixing equal volumes of 2 N pyridine and 2 N sulfuric acid. The acidified ethyl acetate for solvolysis was prepared by saturating redistilled ethyl acetate (10 vol.) with 2 M sulfuric acid (1 vol.) in a separatory funnel[10]. The *N*-O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from the Pierce Chemical Co. (Rockford, Ill., U.S.A.). *Helix pomatia* digestive juice was purchased from l'Industrie Biologique Française (Gennevilliers, France). Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The solvent mixtures used for Sephadex LH-20 chromatography were S1: chloroform–heptane–methanol (5:5:1, by vol) and S2: chloroform–methanol (1:2, by vol.)[5].

3. *Steroids.* Except for 5β-cholestane-3α-ol obtained from Sigma Chemical Company (St-Louis, U.S.A.) and for 5β-androstane-3,16α,17β-triol isomers obtained from Steraloïds Inc. (Wilton, U.S.A.), all other reference steroids were purchased from Ikapharm (Ramat-Gan, Israël).

The 5β-pregnane-3α,6α,20α-triol and 5β-pregnane-3α,6α,20β-triol were obtained by reduction of 3α,6α-dihydroxy-5β-pregnan-20-one with sodium borohydride.

4. *Reduction of 3α,6α-dihydroxy-5β-pregnan-20-one.* The steroid (200 μg) was dissolved in methanol (1 ml) and reduced with sodium borohydride (5 mg) for 1 h at room temperature. The reaction was stopped with 0.5 M hydrochloric acid.

Distilled water (2 ml) was added and the methanol evaporated. The reduced steroids were extracted with ethyl acetate (25 ml). The extract was dried by filtration on anhydrous sodium sulfate and evaporated to dryness. The sample was dissolved by sonication in methanol (5 ml), and the solution was evaporated to dryness in a stream of nitrogen. The residue was dissolved in 0.15 ml of pyridine–BSTFA (1:3, v/v). After 30 min at 60°C, the reduced steroids are analysed by gas–liquid chromatography (GLC) and gas liquid chromatography–mass spectrometry (GLC–MS).

5. *Preparation of column for chromatography on Sephadex LH-20.* Glass columns, 50 cm × 0.7 cm i.d., with a 50 ml solvent reservoir and Teflon stopcock were used. Sephadex LH-20 (5 g) was suspended in 25 ml of S1. The mixture was stirred for 15 min, added to the column, and left to stand overnight.

6. *Procedure for the analysis of steroids in urine.* The urinary conjugates of steroids were extracted according to Okerholm *et al.*[9]. The ethyl acetate extracts were taken to dryness. Acidified ethyl acetate (20 ml) was added and the solution was incubated at 39°C for 24 h[10]. Following this solvolysis, the ethyl acetate was taken to dryness and the residue dissolved by sonication in distillated water (20 ml). The pH was adjusted to 5.20 by addition of acetic acid. Sodium acetate buffer (2 ml, pH 5.20, 0.1 M), *Helix pomatia* digestive juice (1000 IU of β-glucuronidase per ml of urine) were added and the sample incubated at 37°C for 48 h, a further 1000 IU of β-glucuronidase was added after the first 24 h. Following hydrolysis, the liberated steroids were extracted twice with ethyl acetate (1:1, v/v) and diethyl ether (1:1, v/v). The combined organic phases were washed with sodium bicarbonate 1 M (5 ml) and distilled water until neutral, dried by filtration on anhydrous sodium sulfate, and evaporated to dryness. The residue was dissolved by sonication in 1 ml of S1 and the solution transferred to the Sephadex LH-20 column. The neutral steroids were eluted with 65 ml of S1 and the polar steroids with 50 ml of S2. During chromatography, the pressure was adjusted to maintain a flow rate approximately 10 ml/h. Twenty fraction (5 ml) were collected. Each fraction was evaporated to dryness and the residue dissolved in 0.15 ml of pyridine–BSTFA (1:3, v/v). After reaction for 30 min at 60°C, the steroid trimethylsilyl ethers were analysed by GLC and GLC–MS.

7. *Gas–liquid chromatography and gas–liquid chromatography–mass spectrometry.* A Becker 409 gas chromatograph equiped with a flame ionisation detector was employed. The columns were 3000 × 3 mm glass with 0.5% OV-1, 0.5% OV-17, 1% SE-30 and 1% QF-1. The flow rate of carrier gas (nitrogen) was 30 ml/min. On OV-1 and OV-17, the temperature was programmed from 180°C to 260°C at a rate of 1.2°C/min. On SE-30 and QF-1, isothermal conditions (230°C) were employed. The temperature of injection port and detector were 245°C and 275°C respectively.

A GLC–MS analysis was carried out using LKB 9000 instrument equipped with a 1% OV-1 column. The flow rate of carrier gas (helium) was 30 ml/min. The temperature of injection port, separator and ion source were 250°C, 280°C and 290°C respectively. Accelerating voltage, energy of bombarding electrons and ionizing current were 3.5 KV, 22.5 eV and 60 μA respectively. The column was programmed from 180 to 260°C at 1°C/min.

8. *Identification and quantitation of steroid.* A steroid was considered as identified when the methylene unit values (MU) on OV-1 and OV-17, the relative retention times (RT) on SE-30 and QF-1 and the mass spectrum were identical with those of the reference compound. After determination of response coefficient of different steroids, the quantity of steroids present in the different fractions was obtained by addition of known amount of 3α-hydroxy-5β-cholestane (25 to 100 μg) to the samples prior to TMS-derivative formation.

RESULTS AND DISCUSSION

1. *Gas chromatography and gas chromatography–mass spectrometry analysis of reference steroids as silyl ethers*

The mass spectrum of 3α,6α-dihydroxy-5β-pregnan-20-one silyl ether (MU = 27.52 on OV-1) gave a molecular ion (M) at $m/e = 478$, a base peak at $m/e = 298$: M-2 × 90 and prominent peaks at $m/e = 388$: M-90, 299: M-(90 + 89), 251 and 161. For this stereoisomer peaks at $m/e = 204$, 213, 243, 283 and 334 were found with intensities less than 20%.

Analysis by GLC of steroids silyl ethers obtained after reduction of 3α,6α-dihydroxy-5β-pregnan-20-one with sodium borohydride showed three peaks with MU values at 27.53, 28.59 and 28.91 on OV-1. Analysis by GLC–MS indicated that the first peak corresponded to the unreduced 3α,6α-dihydroxy-5β-pregnan-20-one silyl ether. The mass spectra of the second and third chromatographic peaks (Fig. 1) were very similar: molecular ion (M) was at $m/e = 552$, the base peak was at $m/e = 117$ (characteristic of 20-trimethylsilyloxy-21-deoxysteroids) and the prominent peaks were at $m/e = 537$: M-15, 462: M-90, 372: M-(2 × 90), 355: (M-2 × 90 + 15), 282: M-(3 × 90), 267: M(3 × 90 + 15) and 256. This fragmentation pattern indicated that the two reduced steroids obtained from 3α,6α-dihydroxy-5β-pregnan-20-one were isomeric (20α and 20β) tris-trimethylsilyloxy derivatives. The two compounds were identified as 5β-pregnane-3α,6α,20β-triol and 5β-pregnane-3α,6α,20α-triol respectively, the former represented 90% and the latter 10% of the

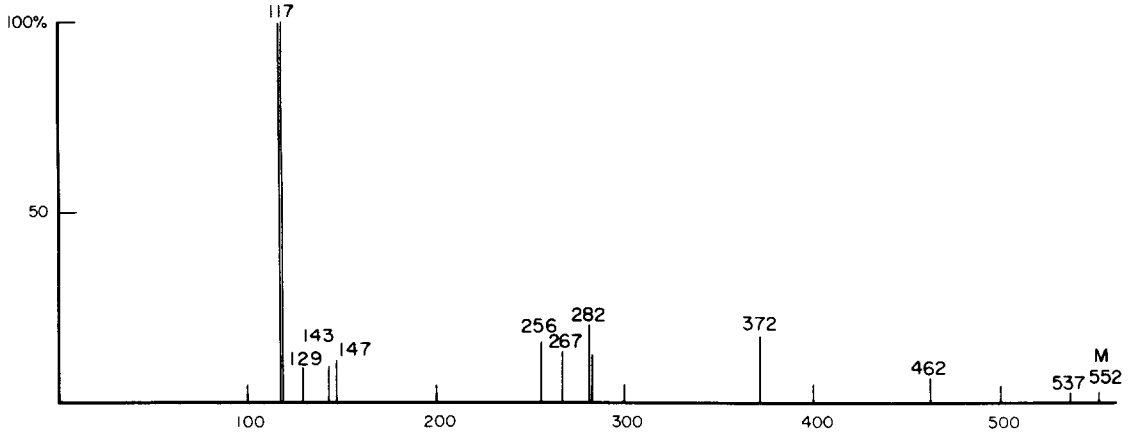

Fig. 1. Mass spectrum of the TMS ether of 5β-pregnane-3α,6α,20α-triol (MU = 28.92 on OV-1) obtained after sodium borohydride reduction of 3α,6α-dihydroxy-5β-pregnan-20-one.

Table 1. MU values on 0.5% OV-1 and 0.5% OV-17 and relative retention times (5α-cholestane = 1.00) on 1% SE-30 and 1% QF-1 of silyl ethers of reference steroids: 3α,6α-dihydroxy-5β-pregnan-20-one, 5β-pregnane-3α,6α,20β-triol and 5β-pregnane-3α,6α,20α-triol

Reference compound	MU/OV-1	MU/OV-17	RT/SE-30	RT/QF-1
3α,6α-Dihydroxy-5β-pregnan-20-one	27.52	29.33	0.92	1.74
5β-Pregnane-3α,6α,20β-triol	28.59	28.72	1.21	0.91
5β-Pregnane-3α,6α,20α-triol	28.91	28.93	1.33	1.00

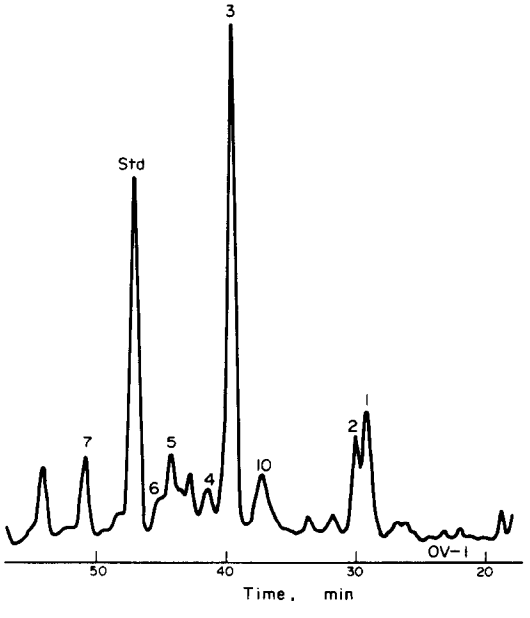

Fig. 2. Gas chromatographic analysis (OV-1 column) of silyl ethers of steroids isolated in the Sephadex LH-20 fraction 8 of urine from normal pregnant women. The numbered compounds were identified as the silyl ether of 5β-androstane-3β,16α,17β-triol (compound 1), 5β-pregnane-3α,20α-diol (compound 2), 5β-pregnane-3α,6α,20α-triol (compound 3), 5ξ-prenane-2ξ,3ξ,20ξ-triol (compound 4), 2ξ,3ξ,16ξ-trihydroxy-5ξ-pregnan-20-one (compound 5), 5α-pregnane-3β,16α,20α-triol (compound 6) and 5-pregnane-3β,16α,20α-triol (compound 7).

products. A trace of 3,6,20-enol-TMS ether of 3α,6α-dihydroxy-5β-pregnan-20-one was eluted on OV-1 with the 5β-pregnane-3α,6α,20β-triol silyl ether. Table 1 shows the MU values and relative retention times of the three reference compounds as TMSi derivatives.

2. *Gas chromatography and gas chromatography–mass spectrometry analysis of urine from pregnant women*

Figure 2 shows the gas chromatographic analysis of the steroids isolated in Sephadex LH-20 fraction 8 from urine of pregnant women during the second trimester of gestation (26 weeks). In Sephadex LH-20 fraction 8 seven distinct steroids were identified by GLC and GLC–MS. The compounds 1 and 2 have been identified as 5β-androstane-3β,16α,17β-triol and 5β-pregnane-3α,20α-diol respectively. The last compound was principally eluted in Sephadex fraction 4 and 5. The mass spectrum of the silyl ether of the compound 3 (Fig. 3) was identical to those of 5β-pregnane-3α,6α,20-triol silyl ethers. In addition, the MU values on OV-1 and OV-17 and the relative retention times on SE-30 and QF-1 of the silyl ether of this compound were identical to those determined for the authentic 5β-pregnane-3α,6α,20α-triol silyl ether (Table 2). According to gas chromatographic and mass spectrometric data, the compound 3 was identified as 5β-pregnane-

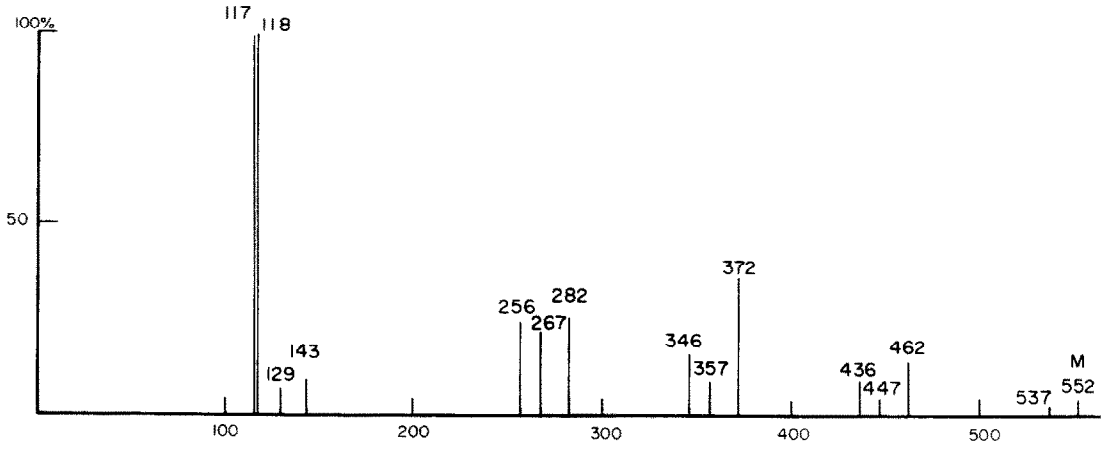

Fig. 3. Mass spectrum of the TMS ether of compound 3 isolated in the Sephadex LH-20 fraction 8, and identified by GLC as 5β-pregnane-3α,6α,20α-triol.

3α,6α,20α-triol. The compounds 4, 5, 6 and 7 eluted in Sephadex LH-20 fraction 8 were identified by GLC–MS as 5ξ-pregnane-2ξ,3ξ,20ξ-triol[12], 2ξ,3ξ,16ξ-trihydroxy-5ξ-pregnan-20-one[12], and by GLC and GLC–MS as 5α-pregnane-3β,16α,20α-triol and 5-pregnene-3β,16α,20α-triol[13].

GLC and GLC–MS analysis of urinary steroids isolated from Sephadex LH-20 fraction 9 demonstrated the presence of the 5β-androstane-3β,16α,17β-triol, 5β-pregnane-3α,6α,20α-triol, and 5-pregnene-3β,16α,20α-triol mentioned above. Three additional compounds: 16α-hydroxyestrone (eluted with a trace of 5ξ-androstane-2ξ,3ξ,17ξ-triol on OV-1), 16-oxoestradiol[14] and another isomer of 5-pregnane-3,6,20-triol were also identified in this fraction. The mass spectrum of this compound is identical to that of 5β-pregnane-3α,6α,20-triol silyl ethers. The difference between the MU values for this compound and 5β-pregnane-3α,6α,20α-triol was identical to that calculated for 5α and 5β-pregnane-3α,20α-diol. Based on these results, this compound was tentatively identified as 5α-pregnane-3α,6α,20α-triol[4]. However, in the present study compared with the excretion of the 5β isomer, that of 5α-pregnane-3α,6α,20α-triol was relatively low (Ca 5%). MU values, relative retention times and characteristic fragmentations of the different compound as silyl ethers isolated from Sephadex LH-20 fraction 8 and 9 are shown in Table 2.

In the present investigation, 5β-pregnane-3α,6α,20α-triol was isolated, identified and measured in urine from 15 normal pregnant women. This compound was not detected before the 16th week of gestation. After this time, this metabolite was found in a significant quantity (0.20 to 2.90 mg/24 h) in all pregnancy urines analyzed. In spite of large individual variations in the excretion of 5β-pregnane-3α,6α,20α-triol, especially between the 16th and 26th week, a significant increase in excretion of this compound was observed during the following five weeks (26th to 30th). Mean values in Table 3 are 16th to 26th week = 0.8 mg/24 h, 26th to 30th = 2.2 mg/24 h. After this time, the excretion of the 5β-pregnane-3α,6α,20α-triol appeared to increase from 30th week until 36th week. However, this increase was not quantitatively significant. In contrast the excretion of other catabolites of progesterone reaches maxima levels between the 30 to 36 week of the gestation and then decreases until parturition. These results confirm those found by James *et al.*[8] since these authors showed that during the last 10 weeks of pregnancy the urinary excretion of 5β-pregnane-3α,20α-diol was relatively constant whereas excretion of 6-oxygenated metabolites of progesterone continued to increase from week 10 until 4 weeks before the delivery. After this time, excretion was practically constant. In the work mentioned above, the mean values found for excretion of total 6-oxygenated metabolites during the last 12 weeks of pregnancy were between 3.7 and 7.2 mg/24 h. The difference observed between those values and our results may be explained by the fact that in the present work 5β-pregnane-3α,6α,20α-triol was assayed specifically. Comparison of our values with those found by James *et al.*, showed that this compound represents about 30% of the total excretion of 6-oxygenated metabolites of progesterone.

These *in vivo* results together with those obtained especially by Fotherby[1, 4, 6–8], confirmed that during human gestation, the excretion of 6α-hydroxy catabolites of progesterone is relatively important. In this paper, the pattern of excretion of 5β-pregnane-3α,6α,20α-triol in the urine of pregnant women was followed from the 16th week of gestation up to time of delivery. In the course of these studies, it was found that excretion of 5β-pregnane-3α,6α,20α-triol may approach 3 mg/24 h at 34 and 35 weeks of the gestation. It is also shown that the excretion pattern of this compound exhibits a marked increase between the 26th and 30th week of gestation. Our *in vivo* results can not explain this fact since the biological

Table 2. Gas chromatographic and mass spectrometric characteristics of silyl ethers of metabolites identified in the Sephadex LH-20 fraction 8 and 9

	Fraction LH-20	MU/OV-1	RT/SE-30	RT/QF-1	M+	Predominant peaks
5β-Androstane-3β,16α,17β-triol Compound 1	8–10	27.35	0.84	0.67	524	191-239-344-419-434-509
5β-Pregnane-3α,20α-diol Compound 2	4–8	27.41	0.94	0.87	464	117-269-284-449
16α-Hydroxyestrone Compound 8	9–11	27.40	0.91	1.75	430	218-231-244-271-286-342-415
16-Oxoestradiol Compound 9	9–11	27.66	1.00	2.28	430	129-143-245-258-271-285-312-402-430
5α-Pregnane-3α,6α,20α-triol* Compound 10	9–10	28.58	—	—	552	117-256-267-282-372-462
5β-Pregnane-3α,6α,20α-triol Compound 3	8–9	28.93	1.33	1.00	552	117-256-267-282-372-462
5ξ-Pregnan-2ξ,3ξ,20ξ-triol Compound 4	6–8	29.20	1.49	1.16	552	117-129-142-143-232-267-282-372-462-537
2ξ,3ξ,16ξ-Trihydroxy-5ξ-pregnan-20-one Compound 5	7–8	29.59	1.62	2.02	566	96-109-129-142-143-157-159-172-186-253-296-386-476-551
5α-Pregnane-3β,16α,20α-triol Compound 6	6–8	29.76	1.71	1.30	552	117-141-155-157-462
5-Pregnene-3β,16α,20α-triol Compound 7	8–9	30.65	—	—	550	117-129-141-156-157-460

Table 3. Urinary levels (mg/24 h) of estriol and the principal catabolites of progesterone are included with the values obtained for 5β-pregnane-3α,6α,20α-triol in this study of pregnant women at different stages of gestation as indicated by the numbers in parentheses

Steroid	Bor. (16)	Sus. (20)	Pois. (24)	Laf. (24)	Mar. (24)	Fou. (26)	Ker. (26)	Jul. (30)	Des. (32)	Mor. (34)	Lev. (36)	Mon. (38)	Fra. (40)	Pie. (34)	Che. (35)
5β-Pregnane-3α,6α,20α-triol	0.75	0.65	1.55	0.25	0.20	0.95	0.35	2.15	1.90	2.35	2.50	2.15	2.10	2.90	2.10
3α-hydroxy-5β-pregnan-20-one	5.90	3.65	3.90	5.80	5.20	3.20	4.85	12.10	6.65	10.10	13.30	7.10	7.10	18.0	17.0
5α-Pregnane-3α,20α-diol	0.70	1.65	1.30	2.45	3.00	0.90	4.40	5.20	3.75	4.50	5.15	4.20	—	—	13.50
3α,16α-Dihydroxy-5β-prégnan-20-one	3.45	2.30	3.50	3.20	4.30	4.40	4.10	7.90	5.20	6.95	12.30	4.30	6.20	11.70	23.00
5β-Pregnane-3α,20α-diol	31.10	16.80	28.90	14.40	15.50	24.00	32.00	45.90	43.60	31.30	46.80	31.80	43.50	60.50	36.00
5β-Pregnane-3α,17,20α-triol	2.15	1.65	1.55	2.40	2.10	1.85	2.05	3.20	3.60	2.60	4.00	3.20	3.00	6.60	3.80
Oestriol	11.70	8.40	10.90	17.90	14.60	19.10	15.60	17.70	14.50	19.00	40.70	21.50	24.10	37.50	48.00

formation of 6-oxygenated metabolites of progesterone has not been completely elucidated. It is probable that the immediate precursor of this compound was the 3α,6α-dihydroxy-5β-pregnan-20-one, since this last compound was also found in urine of pregnant women. Possibly, the excretion of 5β-pregnane-3α,6α,20α-triol can reflect the maternal metabolism of the 6α-hydroxyprogesterone formed (with 20α-di-hydroprogesterone and 6β-hydroxyprogesterone) in the placenta from progesterone[15, 16]. According to this hypothesis, the enzymes involved in the formation of 5β-pregnane-3α,6α,20α-triol were the 5β-reductase, 3α-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase. However, it is not possible to specify either the exact (or predominant) enzymic pathway, since the enzymes mentioned above are present in both the maternal and fetal comportments. In addition to placenta, the progesterone can also be hydroxylated in position 6α (and 6β) by human liver microsomes[17], human fetal liver[18] and fetal rat liver cells in culture[9]. Finally, since the physiopathological importance of the excretion of 6α-hydroxy-21-deoxysteroids *in vivo* is not completely known we intend to pursue and complete the study of these compounds in the urinary samples from abnormal pregnancies.

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REFERENCES

1. James F., Fotherby K. and MacNaugton M. C.: [4-^{14}C] Progesterone metabolism in women during pregnancy. *Endocrinology* **55** (1969) 34–42.
2. Lieberman S., Dobriner K., Hill B. R., Fieser L. F. and Rhoads C. P.: Studies in steroid metabolism. II. Identification and characterization of ketosteroids isolated from urine of healthy and diseased persons. *J. biol. Chem.* **172** (1948) 263–295.
3. Salamon I. I. and Dobriner K.: 3β,6α-Dihydroxyallopregnane-20-one from human urine. *J. biol. Chem.* **207** (1954) 323–330.
4. Fotherby K.: Metabolism of progesterone to 6-oxygenated steroids *in vivo* and *in vitro*. *Adv. Biosci.* **3** (1968) 43–50.
5. Bègue R. J., Desgres J., Gustafsson J. Å. and Padieu P.: Analyse modulaire des stéroïdes urinaires pendant la gestation humaine. *J. steroid Biochem.* **7** (1976) 211–221.
6. James F. and Fotherby K.: A method for the estimation of 6-oxygenated metabolites of progesterone in urine. *Biochem. J.* **95** (1965) 459–465.
7. Fotherby K., James F., Kamyab S., Klopper A. I. and Wilson G. R.: Excretion of 6-oxygenated metabolites of progesterone and 5β-pregnane-3α,17α,20α-triol during pregnancy. *J. Endocr.* **33** (1965) 133–143.
8. James F., Fotherby K. and Klopper A. I.: Excretion of 6-oxygenated metabolites of progesterone and pregnanediol during pregnancy. *Clin. Endocr. (Oxford)* **1** (1972) 73–79.
9. Okerholm R. A., Chattoraj S. C., Pinkus J. L., Charles D. and Wotiz H. H.: Quantitative extraction of steroid conjugates from urine by use of a liquid–liquid partition system. *Steroids* **16** (1970) 66–68.
10. Vihko R.: Gas chromatographic–mass spectrometric studies on solvolyzable steroids in human peripheral plasma. *Acta endocr., Copenh. Suppl.* **109** (1966) 1–67.
11. Laatikainen T.: Identification of $C_{19}O_3$ steroids in the mono and disulphate fractions of human bile. *Steroids* **15** (1970) 139–150.
12. Eriksson H., Gustafsson J. Å. and Poussette Å.: Metabolism of androstenedione and progesterone in the isolated perfused rat liver. *Eur. J. Biochem.* **27** (1972) 327–334.
13. Gustafsson J. Å., Lisboa B. P. and Sjövall J.: Steroids in newborns and infants. C_{19} and C_{21} steroids in faeces from infants. *Eur. J. Biochem.* **10** (1969) 302–311.
14. Adlercreutz H. and Luukkainen T.: Biochemical and clinical aspects of the enterophepatic circulation of estrogens. *Acta endocr. Copenh. suppl.* **124** (1967) 101–140.
15. Kitchin J. D., Pion R. J. and Conrad S. H.: Metabolism of progesterone by term human placenta perfused *in situ*. *Steroids* **9** (1967) 263–274.
16. Jaffé R. B. and Ledger W. J.: *In vivo* steroid biogenesis and metabolism in human term placenta. *Steroids* **8** (1966) 61–78.
17. Björkhem I., Einarsson K., Gustafsson J. Å. and Somell A.: Metabolism of 3β-hydroxy-Δ^5- and 3-oxo-Δ^4-C_{19} and C_{21} steroids in human liver microsomes. *Acta endocr., Copenh.* **71** (1972) 569–588.
18. Ingelman-Sundberg M., Rane A. and Gustafsson J. Å.: Properties of hydroxylase systems in the human fetal liver. Active on free and sulfoconjugated steroids. *Biochemistry* **14** (1975) 429–437.
19. Desgrès J., Guiguet M., Bègue R. J. and Padieu P.: Study of progesterone metabolism in fetal and postnatal rat liver cells in culture. In *Advances in Mass Spectrometry in Biochemistry and Medicine* (Edited by A. Frigerio and N. Castagnoli). Spectrum Publications, New York (1976) pp. 139–155.