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IDENTIFICATION AND QUANTITATION OF 16α-HYDROXY C_{21} STEROID SULPHATES IN PLASMA FROM PREGNANT WOMEN

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SUMMARY

Plasma steroids from 11 women in the last trimester of pregnancy were separated into conjugate class by chromatography on Sephadex LH-20. Fractions corresponding to mono- and disulphate conjugates were solvolyzed and the free steroids were separated by lipophilic gel chromatography into groups containing mono-, di- and trihydroxy steroids, respectively. Trimethylsilyl ether derivatives were prepared and repetitive-scanning gas chromatography–mass spectrometry (GC–MS) was used to characterize and quantitate the following 16α-hydroxy steroids in the monosulphate fraction: 3α,16α-dihydroxy-5α-pregnan-20-one (range 8–80 ng/ml), 3β,16α-dihydroxy-5α-pregnan-20-one (6–55 ng/ml), 5α-pregnane-3α,16α,20α-triol (9–31 ng/ml) and 5α-pregnane-3β,16α,20α-triol (11–77 ng/ml). These compounds were also present as disulphates although at 5–50 times lower concentrations.

INTRODUCTION

During pregnancy, sulphate esters represent a quantitatively important class of steroid derivatives both in the fetal and maternal compartments [1–3]. The major sulphoconjugated steroids in the fetus possess a 3β-hydroxy-5-ene structure and a hydroxyl group in the 16α-position [4, 5]. In contrast, the sulphoconjugated steroids in the maternal compartment are predominantly saturated pregnane derivatives, unsubstituted at C-16 [6]. In a recent study of the turnover in maternal plasma of these compounds [7], evidence was obtained for the formation of 16-hydroxylated metabolites. Although the fetal liver has a very high 16-hydroxylating activity [8], liver microsomes from adult humans can also carry out hydroxylation of neutral steroids in the 16α-position, employing both sulphates and unconjugated steroids as substrates [9]. Since it has been suggested that the determination of 16-hydroxylated neutral steroids in the urine of pregnant women may yield information about the functional state of the feto-placental unit [10], it is important to establish to what extent the hepatic metabolism of sulphated precursors in the maternal compartment contributes towards the production of these 16-hydroxysteroids.

The present paper deals with the initial part of such an investigation and describes the isolation, characterization and measurement of 16-hydroxy C_{21} steroid sulphates from plasma of healthy women in the last trimester of pregnancy.

EXPERIMENTAL

Analytical grade solvents were redistilled once for use in the extraction of plasma samples and twice for use in Lipidex^R-5000 gel chromatography.

Glassware was cleaned in an ultrasonic bath and was used for plasma samples only.

Reference samples of steroids were of commercial origin except for 3α,16α-dihydroxy-5α-pregnan-20-one and 5α-pregnane-3α,16α,20α-triol which were gifts from Dr. R. Neher, and 3β,16α-dihydroxy-5α-pregnan-20-one and 3β,16α-dihydroxy-5β-pregnan-20-one, which were donated from the MRC Reference Steroid Collection by Prof. W. Klyne.

Lipidex^R-5000 was purchased from Packard International SA, Zürich.

Plasma samples. Blood from healthy women in the last trimester of pregnancy (Table 2) was collected in heparinized tubes. The samples were immediately centrifuged and the plasma was stored at −18°C until analyzed. Samples from 15 healthy subjects were used as a pool.

Plasma was extracted with ethanol/acetone and the extract was separated on a column of Sephadex LH-20 into steroid mono- and disulphate fractions [11]. Following solvolysis and purification on silicic acid, steroids were separated by chromatography on Lipidex^R-5000 or taken directly for derivatization. The volume of plasma used for quantitative analyses was 2–3 ml.

Lipidex^R-5000 gel chromatography was carried out using silanised glass columns (30 × 1 cm.), equipped with a solvent reservoir and a tap and calibrated in ml to permit measurement of the gel bed vol. Lipidex^R-5000 (6 g) was equilibrated in the eluant (hexane–chloroform–methanol, 85:15:1, by vol.) for 30

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min before packing the column under gravity flow to give a bed vol. of approximately 24 ml. Immediately before application of the sample the column was washed with 3 5 bed vol. of eluant, with the tap fully open. The plasma steroids were dissolved in 32 μl of chloroform–methanol, 15:1 (v/v), and 170 μl of hexane were added. The solution was applied to the column and the process was repeated twice. Elution was carried out at atmospheric pressure and room temperature with a flow rate of 15–20 ml/h. When reference compounds were chromatographed, 2 ml fractions were collected in calibrated tubes and the steroids were detected by thin-layer chromatography. For each substance the standard elution volume [12] was calculated.

$$\text{Standard Elution Volume (SEV)} = \frac{\text{Elution Volume of Steroid}}{\text{Bed Volume}} \times 100$$

For group separations of plasma steroids, large fractions were collected in measuring cylinders. After the elution of the dihydroxy steroids (i.e. after SEV 525) the eluant was changed to methanol and a further 25 ml of solvent were collected with the tap fully open. This fraction contained polar steroids with three or more hydroxyl functions.

Trimethylsilyl (TMS) ethers were prepared according to Makita and Wells[13] using redistilled hexamethyldisilazane, trimethylchlorosilane and dry pyridine in proportions 2:1:3 (by vol.). For quantitative analyses an internal standard, 5β-cholanc-3α,24-diol (125 ng) was added prior to derivative formation.

Gas–liquid chromatography (g.l.c.) was performed on 1·5% SE-30 and HiEff 8 BP columns using a Pye Series 104 instrument equipped with flame ionization detectors. Retention times (t_R) of steroid TMS ethers were recorded relative to that of 5α-cholestane (t_R = 1·0).

Gas chromatography–mass spectrometry (GC–MS) was carried out with a modified LKB 9000 instrument [14], using an electron energy of 22·5 eV, source temperature 290°C and accelerating voltage of 3·5 kV. Spectra were taken at intervals of 6 s (repetitive magnetic scanning) and were recorded on magnetic tape. Quantitative analyses were carried out using repetitive accelerating voltage scanning over a range of 8 mass units and with a scan time of 5 s. Data were evaluated by off-line processing on an IBM 1800 computer.

Computer programs. Descriptions of programs MINIG and ION4X have already been published [15]. Program AREAT, a modified version of program AREAS, was used to calculate the areas of peaks in fragment ion current (FIC) chromatograms constructed by ION4X.

RESULTS

Lipidex^R gel chromatography. The chromatographic system employed in this study was first evaluated with reference samples of the major steroids found in the sulphate fractions from pregnancy plasma. Fractions of effluent (range 10 SEV units) were analyzed by t.l.c. and g.l.c. and the elution zone for each compound determined (Table 1). On the basis of these values, the fraction boundaries were established for the group separation of C-21 steroids containing one, two and three or more hydroxyl functions as SEV 100–225, 225–525 and 525+, respectively. These SEV boundaries were verified using extracts of steroids from plasma of pregnant women. Elution volumes for individual steroids in this chromatographic system have been found to be highly reproducible and the method is currently in routine use in our laboratory for the separation of unconjugated steroids.

Identification of steroids. Mono- and disulphate fractions obtained by Sephadex LH-20 chromatography of the steroid extract from 10 ml of pregnancy plasma were solvolyzed and the free steroids were separated on the Lipidex gel. Steroids in the "dihydroxy" (225–525 SEV) and "trihydroxy" (>525 SEV) fractions were trimethylsilylated and analyzed by g.l.c. and by repetitive scanning GC–MS. Relative retention times, t_R, on SE-30 and HiEff 8 BP stationary phases of the steroids found are given in Table 1. Fragment ion chromatograms were constructed (program MINIG) for those ions characteristic of the trimethylsilyl ether derivatives of 3,16-dihydroxypregnan-20-ones, at *m/e* 159, 172, 186, 255, 283, 345 and 388 [16] and of pregnane-3,16,20-triols, at *m/e* 117, 141, 156, 157, 447 and 462 [17] (Figs. 1 and 2).

Criteria for the identification of the 16-hydroxylated pregnanolone and pregnanediol derivatives were two-fold: (a) coincident peaks were present in the respective sets of fragment ion chromatograms, having the same retention times on both stationary phases as those of the authentic reference steroids; (b) the relative intensities of the characteristic ions in each case were the same as those found in the mass spectra of the reference compounds. On the basis of these criteria, the following 16-hydroxysteroids were identified: 3α,16α-dihydroxy-5α-pregnan-20-one, 3β,16α-dihydroxy-5α-pregnan-20-one, 5α-pregnane-3α,16α,20α-triol and 5α-pregnane-3β,16α,20α-triol. Criterion (a) was insufficient to distinguish 3α,16α-dihydroxy-5α-pregnan-20-one from the isomeric 3β,16α-dihydroxy-5β-pregnan-20-one. However, differences in relative intensities of several major fragment ions (*m/e* 255, 283, 298, 373, 388) in the mass spectra of the TMS ethers of these compounds permitted the unambiguous assignment of the 3α,5α-structure. Each compound was present in both the mono- and disulphate fractions from the LH-20 column and had the same mobility as the reference compounds during Lipidex^R chromatography.

Figure 1 illustrates the presence of the two isomeric 16-hydroxylated pregnanolones in the "dihydroxy" fraction from Lipidex^R chromatography of the plasma monosulphate sample. Similarly, Fig. 2 shows the co-occurrence of the corresponding pregnane-

Table 1. Chromatographic data for steroids from pregnancy plasma

Compound	MoS	DiS	t_R (TMS ether) SE-30	t_R (TMS ether) HiEff 8 BP	SEV range Lipidex^R-5000
3α-Hydroxy-5α-androstan-17-one	+	-	0.37		
3β-Hydroxy-5-androsten-17-one	+	-	0.49	1.19	210-250
5-Androstene-3β,17α-diol	-	+	0.54	0.33	
5-Androstene-3β,17β-diol	-	+	0.61	0.41	
3α,16α-Dihydroxy-5-androsten-17-one[a]	+	-	0.62		
3β,16α-Dihydroxy-5-androsten-17-one	+	-	0.86		
3β,17β-Dihydroxy-5-androsten-16-one	+	-	0.93		
5-Androstene-3β,16α,17α-triol	+	-	1.02		
5-Androstene-3β,16α,17β-triol	+	-	1.22		
3α-Hydroxy-5α-pregnan-20-one	+	-	0.64	1.11	100-200
3α-Hydroxy-5β-pregnan-20-one	+	-	0.64	1.48	
3β-Hydroxy-5-pregnen-20-one	+	-	0.83	1.85	100-200
3β-Hydroxy-5α-pregnan-20-one	+	-	0.83	1.80	100-200
5α-Pregnane-3α,20α-diol	+	+	0.93	0.53	225-400
5β-Pregnane-3α,20α-diol	+	+	0.93	0.69	
3α,16α-Dihydroxy-5α-pregnan-20-one	+	+	1.02	1.01	450-525
5-Pregnene-3β,20α-diol	+	+	1.17	0.84	275-450
5α-Pregnane-3β,20α-diol	+	+	1.17	0.83	250-450
3β,16α-Dihydroxy-5α-pregnan-20-one	+	+	1.31	1.63	450-525
5α-Pregnane-3α,16α,20α-triol	+	+	1.42	0.51	525-600
3α,21-Dihydroxy-5α-pregnan-20-one[a]	+	-	1.51		225-300
5-Pregnene-3β,16α,20α-triol[a]	+	-	1.83		
5α-Pregnane-3β,16α,20α-triol	+	+	1.85	0.81	525-650
5α-Pregnane-3α,20α,21-triol	+	+	1.89	0.77	>650

[a] Tentative structure.

3,16,20-triols in the "trihydroxy" fraction from the same sample.

Quantitative measurements. Monosulphate and disulphate fractions were obtained by LH-20 chromatography of both pooled pregnancy plasma and of samples from eleven individual subjects. Following solvolysis, addition of internal standard and derivative formation, the four 16-hydroxylated steroids were analyzed by GC–MS using repetitive accelerating voltage scanning. Partial mass spectra were taken during appropriate retention time intervals to monitor the elution of the steroids of interest. The spectra were centered on the ions at *m/e* 388 (3,16-dihydroxy-pregnan-20-ones; M-90), *m/e* 462 (pregnane-3,16,20-triols; M-90) and *m/e* 257 (internal standard). The elution profile of each of the above steroids was examined by means of computer-drawn fragment ion current chromatograms (program ION4X) (Fig. 3). Similar analyses were carried out, under identical recording conditions, of a series of reference mixtures comprising known amounts of 3β,16α-dihydroxy-5α-pregnan-20-one, 5α-pregnane-3β,16α,20α-triol and a constant amount (125 ng) of the internal standard. Areas of peaks in fragment ion chromatograms (program AREAT) were used to construct curves of response versus mass of steroid injected (Fig. 4). A second set of calibration curves was generated using peak height measurements (program ION4X). Since the ion yield

Table 2. Data on subjects

Subject[a]	Age (years)	Week of pregnancy sampling	delivery	Child sex	weight (g)
M.A.	38	39	39	male	3,670
L.H.	31	39	39	female	2,930
M.W.	31	34	37	female	2,710
N.W.	34	40	40	male	3,430
D.Ö.	31	39	39	female	2,830
T.V.	25	40	40	female	2,800
O.M.	29	40	40	male	4,040
E.V.	29	38	40	male	3,880
I.B.	25	37	42	female	2,790
G.J.	26	31	40	male	3,270
I.S.	27	33	40	male	3,020

[a] Patient M.W. was hospitalized in the 29th week of pregnancy on account of abdominal pain arising from *leiomyoma uteri*. Patients G.J. and I.S. were kept in hospital under observation due to suspected incompetent cervix. The other subjects were apparently healthy.

from corresponding 3α- and 3β-isomers was assumed to be the same, and since the same percentage of total ion current resided in the fragment ions used for the measurements, these curves were also used for quantitation of the 3α-isomers. Plasma concentrations of each steroid were calculated from both sets of curves and the results are summarized in Table 3. No corrections were made for losses prior to GC–MS analysis (about 20% [2]).

The plasma concentrations of all the steroids measured were higher in the monosulphate than in the disulphate fractions. Furthermore, 3α,16α-di-

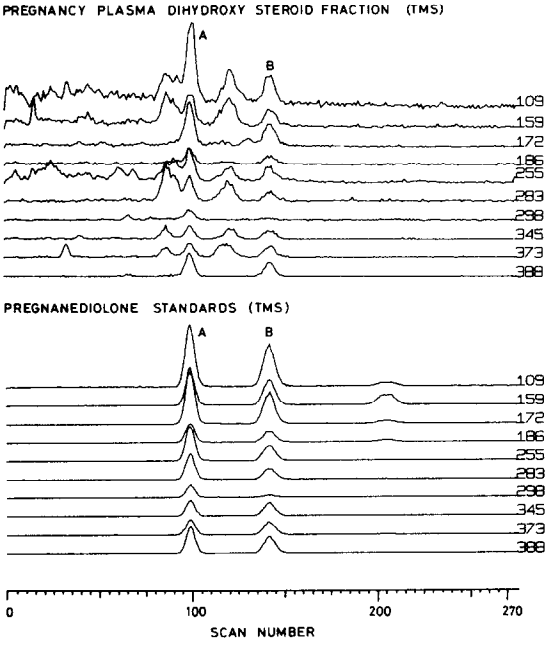

Fig. 1. Fragment ion current (FIC) chromatograms constructed by program MINIG for ions characteristic of 16-hydroxy-20-ketosteroid TMS ether derivatives. The upper set of traces was obtained from an analysis of dihydroxysteroids from the monosulphate fraction of pregnancy plasma, and the lower set from an analysis, carried out under identical conditions, of two standards: 3α,16α-dihydroxy-5α-pregnan-20-one (A) and 3β,16α-dihydroxy-5α-pregnan-20-one (B).

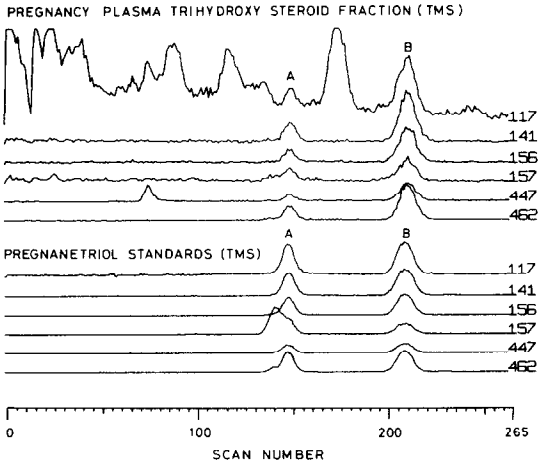

Fig. 2. FIC chromatograms for ions characteristic of pregnane-3,16,20-triol TMS ether derivatives. The upper set of traces was obtained from an analysis of trihydroxysteroids from the monosulphate fraction of pregnancy plasma, and the lower set from an analysis (under identical conditions) of two standards: 5α-pregnane-3α,16α,20α-triol (A) and 5α-pregnane-3β,16α,20α-triol (B). The distortion of the peak in the chromatogram of *m/e* 157 for standard A arises because of a contribution from 3β,16α-dihydroxy-5α-pregnane-20-one present in the mixture of standards.

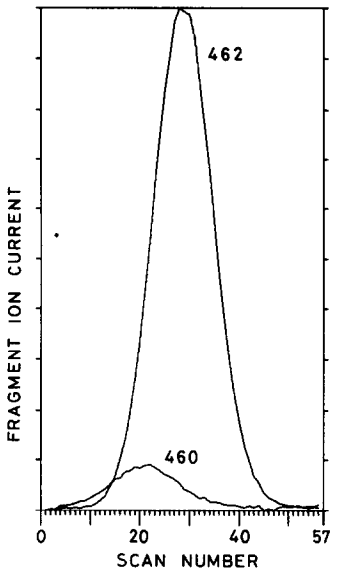

Fig. 3. FIC chromatograms constructed by program ION4X, using data obtained by repetitive accelerating voltage scanning, for the quantitation of 5α-pregnane-3β,16α,20α-triol in the steroid monosulphate fraction of a pregnancy plasma sample. The fragment ion at *m/e* 462 represents (M-90) from this triol, while that at *m/e* 460 probably arises from 5-pregnene-3β,16α,20α-triol.

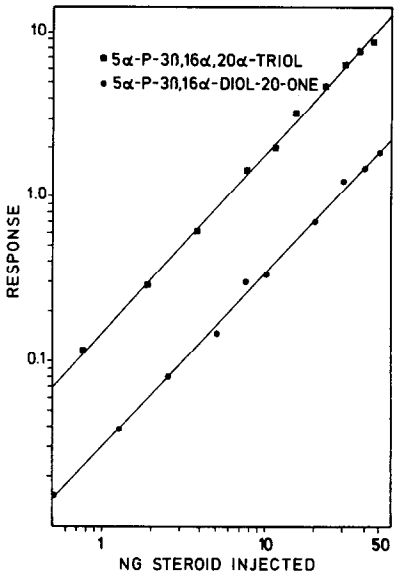

Fig. 4. Calibration curves for the quantitative analysis of 16-hydroxysteroids in pregnancy plasma by repetitive accelerating voltage scanning GC–MS. Response is measured as the peak area in FIC chromatograms of *m/e* 388 (pregnanediolones) or *m/e* 462 (pregnanetriols), relative to that given by 125 ng of the internal standard (*m/e* 257).

hydroxy-5α-pregnan-20-one was the major pregnanediolone in the monosulphate fraction, although it was barely detectable as a disulphate conjugate. In contrast, the major 16-hydroxylated pregnanetriol had a 3β,5α configuration. The concentrations of each of the 16-hydroxysteroid monosulphates were 10–30 times lower than those of their 16-deoxy counterparts (Table 4). The levels of both disulphated pregnane-3,16,20-triols were far below those of the corresponding pregnane-3,20-diol disulphates.

Table 3. Concentrations of 16α-hydroxy steroids (as ng of unconjugated steroid per ml of plasma) in mono- and disulphate fractions of plasma from pregnant women

Subject	5αP-3α,16α-ol-20-one				5αP-3β,16α-ol-20-one				5αP-3α,16α,20α-ol				5αP-3β,16α,20α-ol			
	MoS[a]		DiS		MoS		DiS		MoS		DiS		MoS[a]		DiS	
	A[b]	H[b]	A	H	A	H	A	H	A	H	A	H	A	H	A	H
Pool	33	32	0.9	1.3	22	20	6.4	6.5	22	27	2.3	2.6	40	39	2.5	2.7
M.A.	17	14	-[c]	-	12	8	-	-	13	11	0.8	0.7	32	29	1.9	1.7
L.H.	27	25	-	-	27	23	-	-	21	20	1.4	1.4	77	75	3.1	3.1
D.U.	80	75	0.9	1.2	*[d]	55	9.1	9.2	31	32	4.4	5.5	51	51	6.1	6.9
T.V.	45	41	0.9	0.9	30	23	4.7	3.4	24	22	2.8	2.8	42	41	3.4	3.3
O.M.	12	11	-	-	16	10	4.1	3.5	19	16	1.7	1.7	32	37	2.6	2.0
E.V.	23	24	-	-	18	18	3.8	3.4	16	13	1.7	1.5	56	58	4.8	4.0
I.B.	11	13	-	-	10	11	1.3	1.2	21	18	1.0	1.0	50	53	2.1	2.2
I.S.	12	13	-	-	16	15	1.7	1.5	18	16	0.4	0.3	44	42	3.0	3.0
G.J.	23	22	-	-	23	21	2.1	1.9	24	21	2.8	2.8	45	41	3.0	2.8
M.W.	8	7	-	-	6	6	-	-	9	6	0.7	0.6	11	7	0.8	0.7
N.W.	31	30	-	-	28	25	3.9	2.7	27	27	1.9	1.1	44	47	1.9	2.1

[a] Average of 2 values; [b] Values based on peak area, A, and peak height, H; [c] Less than 0·5 ng/ml; [d] Not calculated because of overlap with an interfering compound.

Table 4. Concentration of 16α-hydroxysteroids as percentage of the corresponding 16-deoxysteroids in mono- and disulphate fractions of plasma from pregnant women

Subject	Per cent 16α-hydroxysteroid					
	3,16α-ol-20-one MoS		3,16α,20α-ol MoS		3,16α,20α-ol DiS	
	3α-ol	3β-ol	3α-ol	3β-ol	3α-ol	3β-ol
N.W.	5.5	5.1	3.7	7.7	0.86	0.76
E.V.	4.0	4.3	3.1	11.0	0.43	0.55
I.S.	3.0	4.6	3.3	8.5	0.09	0.26
I.B.	2.5	10.0	3.7	15.0	0.48	0.48
Mean	3.8	6.0	3.5	10.6	0.47	0.51

DISCUSSION

In the present study, two isomers of 3,16-dihydroxypregnan-20-one and of pregnane-3,16,20-triol, epimeric at C-3, have been characterized in both the mono- and disulphate fractions from plasma of women in the third trimester of pregnancy. Identification of these metabolites was achieved using computerized GC–MS with repetitive magnetic scanning. Coincident peaks in specific fragment ion current chromatograms were employed to detect 3,16-dihydroxypregnan-20-ones and pregnane-3,16,20-triols while the relative retention times on g.l.c. using non-selective (SE-30) and polar (HiEff 8 BP) stationary phases served to define their stereochemistry.

Saturated $C_{21}O_3$ steroids with a 16α-hydroxy group are known to occur in a number of biological fluids, including bile [18], urine [19], faeces [20] and amniotic fluid [21] from pregnant women and meconium [22] and urine [23] from newborn infants. The monosulphates of 3α,16α-dihydroxy-5α-pregnan-20-one [24] and 5α-pregnane-3α,16α,20α-triol [25] have previously been tentatively identified in plasma from healthy pregnant women. However, because of the low concentrations of these steroids and due to the complexity of the "total" monosulphate fraction with many overlapping peaks, the absolute configurations of these compounds could not be unambiguously assigned. The use of lipophilic dextran gels to achieve group separations of free steroids has been shown to be a very promising method for simplifying complex mixtures of biological origin [26, 27]. The commercially available gel, Lipidex^R-5000, was employed in this study to sub-fractionate steroids obtained by solvolysis of the "total" monosulphates and "total" disulphates from pooled pregnancy plasma into groups of steroid diols and triols. This additional purification step afforded samples which gave fewer overlapping peaks in the subsequent GC–MS analysis and thereby facilitated the identification of minor components in the mixture.

The levels of the individual 16-hydroxylated steroids were determined by GC–MS using repetitive accelerating voltage scanning, when quantitation of each compound was based on areas or heights of peaks in specific fragment ion chromatograms. The retention times of the TMS ethers of 5α-pregnane-3β,16α,20α-triol and 5α-pregnane-3α,20α,21-triol on both SE-30 and HiEff 8 BP phases are very similar. However, separation of these positional isomers by Lipidex^R-5000 chromatography and examination of the mass spectra of their TMS ethers showed that only the pregnane-3,16,20-triol gave rise to an ion at *m/e* 462 (M-90). Hence, no interference occurs during quantitation of this steroid in the presence of the pregnane-3,20,21-triol, when the *m/e* 462 fragment is used.

The concentrations of the 16-hydroxylated pregnane derivatives varied considerably between subjects. For example, the level of 5α-pregnane-3β,16α,20α-triol monosulphate, which was usually the major compound of this group, was 11–77 ng/ml plasma. The least abundant member of the group, 3α,16α-dihydroxy-5α-pregnan-20-one disulphate, was usually below the limits for quantitation (less than about 0·5 ng/ml). However, the concentration relationships for 20-keto/20-hydroxy and for 3α-/3β-hydroxy pairs in both mono- and disulphate fractions were similar in each subject. This indicates a specificity in the processes by which the levels of these 16α-hydroxysteroid sulphates are regulated. It is of interest that in pregnant women with intrahepatic cholestasis both the total concentration of steroid sulphates and the ratios between 3α- and 3β-hydroxysteroids are increased. Thus, the monosulphates of 3α,16α-dihydroxy-5α-pregnan-20-one and of 5α-pregnane-3α,16α,20α-triol have been reported to attain levels as high as 530 ng/ml and 300 ng/ml, respectively, in these patients [28].

The physiological significance of the sulphated 16-hydroxy C_{21} steroids in plasma from healthy pregnant women is not known. One of the compounds, 3β,16α-dihydroxy-5α-pregnan-20-one, has

been shown to be active in its unconjugated form in promoting the urinary excretion of sodium ions in rats and is known as the "sodium excreting factor" [29]. Sulphate conjugation of this compound may be important for its "storage", since sulphate esters have a very low renal clearance when compared with the corresponding free steroids [30].

The 16-hydroxysteroids characterized in the present study are possibly metabolites of placental progesterone. However, Ruse and Solomon have shown that direct peripheral 16α-hydroxylation of progesterone does not occur [31], and have obtained evidence that the neutral 16α-hydroxypregnanes in pregnancy urine originate, at least in part, from precursors other than 16α-hydroxyprogesterone [32]. It is not known whether the 16α-hydroxylation takes place in the fetal or maternal compartment. Fetal liver has a high capacity for 16α-hydroxylation, employing as substrates not only unconjugated steroids but also certain steroid sulphates [33, 34]. More recently, however, microsomal preparations from adult human liver have also been shown to carry out direct 16α-hydroxylation of some steroid 3-monosulphates [9]. The concentration in plasma of potential substrates for this hydroxylating system is particularly high during pregnancy. Indeed, recent results from studies on the metabolism in pregnant women of deuterium-labelled steroid sulphates indicate that 16α-hydroxylation of such conjugates does occur under *in vivo* conditions [7].

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