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IDENTIFICATION AND QUANTITATION OF C_{19} AND C_{21} STEROID TRIOL SULPHATES IN ADULT HUMAN URINE

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SUMMARY

Neutral steroid mono- and disulphate fractions were obtained from adult human urine by Sephadex LH-20 chromatography. After solvolysis, the liberated steroids were fractionated on a silicic acid column and by thin-layer chromatography. The following C_{19} and C_{21} steroid triols were identified by gas–liquid chromatography and gas chromatography-mass spectrometry in the monosulphate fraction of urinary steroids: 5α-androstane-3α,16α,17β-triol, 5α-androstane-3β,16α,17β-triol, 5α-androstane-3β,16β,17α-triol, 5α-androstane-3β,16β,17β-triol, 5-androstene-3β,16α,17β-triol, 5-androstene-3β,16β,17α-triol, 5-androstene-3β,16β,17β-triol, 5-pregnene-3β,16α,20α-triol and 5-pregnene-3β,17α,20α-triol. In the disulphate fraction, 5-pregnene-3β,17α,20α-triol and small amounts of 5-androstene-3β,16α,17β-triol were found.

The most abundant compounds in the fraction studied were 5-androstene-3β,16α,17β-triol, 5-androstene-3β,16β,17α-triol and 5-pregnene-3β,17α,20α-triol. Daily excretion of the steroids identified are given for 5 males and 3 females.

INTRODUCTION

THE ROLE of sulphate-conjugated 16-hydroxylated C_{19} steroids in the biosynthesis of oestrogens is well established (summarized in [1]). The capacity of foetal liver to form 16-hydroxylated neutral steroids is high as compared with that of adult liver (reviewed in [2]). However, several 16-hydroxylated ketonic and non-ketonic C_{19} [3–8] and C_{21} steroids [7, 9] have been identified in normal adult urine.

During pregnancy, at least some of the 16-hydroxylated neutral steroids formed by the foetus are transported across the placenta to the maternal circulation and then excreted in the maternal urine [10, 11]. It can be assumed that determination of the maternal excretion of 16-hydroxylated neutral steroids – precursors of oestrogens – will yield information about the functional state of the foeto-placental unit. As a step toward evaluating the significance of the excretion of these oestrogen precursors, we have recently reported the excretion of 16-hydroxylated ketonic C_{19} steroid sulphates in urine of non-pregnant subjects [8]. The present study is a continuation of these investigations and describes the characterisation and measurement of C_{19} steroid triol sulphates in normal female and male urine. In addition, sulphates of two trihydroxylated C_{21} steroids have been identified and measured.

MATERIAL AND METHODS

Reagents. All solvents were of reagent grade and were twice redistilled before use.

Urine was collected for a 24-hr period from 3 normal females and 5 normal males 20–30 years of age. The samples were stored at −20°C until analysed.

Reference steroids. Unless otherwise indicated, the reference steroids were purchased from Ikapharm, Ramat-Gan, Israel. 5α-Androstane-3α,16α,17β-triol and 5β-androstane-3α,16α,17β-triol were supplied by Dr. S. Solomon, Montreal, Canada; 5α-androstane-3β,16α,17β-triol and 5α-androstane-3β,16β,17β-triol by Dr. J. Sjövall, Stockholm, Sweden; and 7α-hydroxydehydroepiandrosterone* by Dr. L. Stárka, Prague, Czechoslovakia. 5-Androstene-3β,16β,17α-triol was obtained by sodium borohydride reduction of 16β-hydroxydehydroepiandrosterone (obtained from Prof. W. Klyne, London, England), and 5α-androstane-3β, 16β,17α-triol by hydrogenation of the corresponding Δ^5-unsaturated steroid. The purity of all reference steroids was checked by thin-layer chromatography, gas–liquid chromatography and gas chromatography-mass spectrometry.

Thin-layer chromatography (TLC) of steroids was carried out using precoated abrasion-resistant Silica Gel F_{254}-layers (Merck AG, No. 5715, 0·25 mm) and the solvent system chloroform: ethanol (9/1, by vol.) with two developments.

Chemical and enzymatic reactions. Hydrogenation of unsaturated steroids was performed using palladium on charcoal as catalyst[12].

Reduction of steroids with sodium borohydride was carried out at room temperature in ethanol overnight.

Oxidation of steroids with 3α-hydroxysteroid dehydrogenase (kindly supplied by Dr. I. Björkhem, Stockholm, Sweden) was made according to Berséus[13].

Gas–liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS). During GLC and GC-MS trimethyl silyl (TMS) ether[14] and O-isopropylidene trimethyl silyl (acetonide-TMS) ether[15] derivatives of steroids were used. GLC and GC-MS were performed on 3% QF-1 and 2·2% SE-30 liquid phases as previously described[16].

Procedure. The details for the isolation of steroid mono- and disulphate fractions from urine have been described previously[8, 17, 18]. After solvolysis[16, 19], the liberated steroids were fractionated on a 3 g column of silicic acid[18]. Fractions eluted with 20 ml of ethyl acetate (silicic acid fraction IV) and 20 ml of methanol (fraction V) were separately subjected to TLC. During the TLC, 16α-hydroxydehydroepiandrosterone and 5-pregnene-3β,17α,20α-triol were used as reference steroids for fraction IV, and 7α-hydroxydehydroepiandrosterone and 5-androstene-3β,16α,17β-triol for fraction V. Relevant zones of the urinary steroids, indicated by the mobility of the reference compounds on the TLC plate, were scraped off and eluted with methanol. Following the preparation of TMS and acetonide-TMS ether derivatives, the steroids were analysed by GLC and GC-MS.

In quantitative analyses, a known amount of stigmasterol (17–35 μg) was added as an internal standard before the formation of the TMS ether derivatives. The peak areas were measured with a planimeter and calculations were made as described previously[16].

**Trivial names:* 16α-Hydroxyandrosterone: 3α,16α-dihydroxy-5α-androstan-17-one; 16α-hydroxydehydroepiandrosterone: 3β,16α-dihydroxy-5-androsten-17-one; 16β-hydroxydehydroepiandrosterone: 3β,16β-dihydroxy-5-androsten-17-one; 7α-hydroxydehydroepiandrosterone: 3β,7α-dihydroxy-5-androsten-17-one; 16α-hydroxyoestrone: 3,16α-dihydroxy-1,3,5(10)-oestratrien-17-one; stigmasterol: (24*R*)-24-ethylcholesta-5,22-dien-3β-ol. The term androstanediol refers to the 8 isomers of 5α/β-androstane-3,17-diol. Similarly, 5-androstene-3,17-diol is a collective name for the isomeric 5-androstene-3,17-diols, androstane-3,16,17-triol for the isomeric 5α/β-androstane-3,16,17-triols, 5-androstene-3,16,17-triol for the isomeric 5-androstene-3,16,17-triols, and oestriol for the isomers of 1,3,5(10)-oestratriene-3,16,17-triol.

RESULTS

In addition to C_{19} and C_{21} steroid triols, silicic acid fractions IV and V contained some other C_{19} steroids[8, 20], and TLC was used to separate neutral steroid triols from other compounds present in these fractions.

Figure 1 shows the GLC analyses of urinary C_{19} and C_{21} steroid triols as the TMS and acetonide-TMS ether derivatives of the monosulphate fraction. In Table 1 the GLC properties of the TMS ether and acetonide-TMS ether derivatives of the compounds identified and of relevant reference compounds are given.

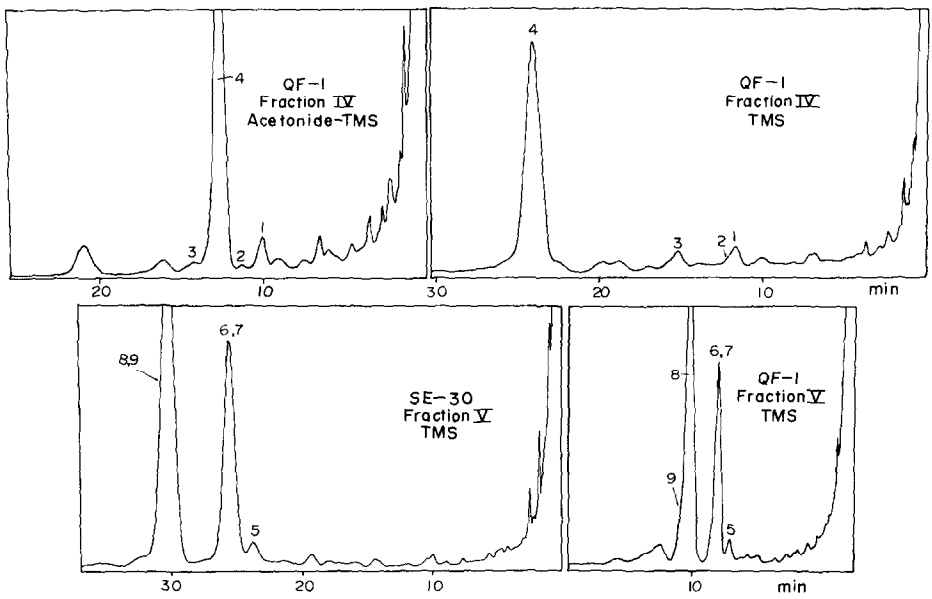

Fig. 1. Gas chromatographic analyses of the TMS and acetonide-TMS ether derivatives of C_{19} and C_{21} steroid triols in the monosulphate fraction of adult human urine. Columns and conditions: 3% QF-1, 2 m × 3·5 mm, 215°C; 2·2% SE-30, 2 m × 3·5 mm, 225°C. Peak identifications: 1 = 5-androstene-3β,16β,17β-triol, 2 = 5α-androstane-3β,16β,17β-triol, 3 = 5-pregnene-3β,16α,20α-triol, 4 = 5-pregnene-3β,17α,20α-triol, 5 = 5α-androstane-3α,16α,17β-triol, 6 = 5-androstene-3β,16β,17α-triol, 7 = 5α-androstane-3β,16β,17α-triol, 8 = 5-androstene-3β,16α,17β-triol and 9 = 5α-androstane-3β,16α,17β-triol.

In the silicic acid fraction IV, the following C_{19} and C_{21} steroid triols were identified:

5-Androstene-3β,16β,17β-triol and 5α-androstane-3β,16β,17β-triol. During GC-MS, the material giving the peak with the RRT (relative retention time, 5α-cholestane = 1·00) values of 1·10 on QF-1 and 1·25 on SE-30 liquid phases (Fig. 1) proved to consist of two steroids (called compounds 1 and 2). The major one had, as the TMS ether derivative, a mass spectrum with a molecular ion at m/e 522 and a fragmentation pattern typical of the corresponding derivative of 5-androstene-3,16,17-triols (for reference spectra, see ref. [21]). The minor compound eluted in the latter part of the peak gave, as the TMS ether, a mass spectrum with a molecular ion at m/e 524 and a fragmentation pattern characteristic of the corresponding derivative of androstane-3,16,17-triols[21, 22]. Each of these two compounds was capable of forming an acetonide, which confirmed the

cis position of the hydroxyl groups in the D-ring[15]. The RRT values of the TMS and acetonide-TMS ether derivatives of reference 5-androstene-3β,16β,17β-triol and 5α-androstane-3β,16β,17β-triol were the same as those of the corresponding derivatives of compounds 1 and 2, respectively (Table 1). These data confirm the identification of these steroids as 5-androstene-3β,16β,17β-triol and 5α-androstane-3β,16β,17β-triol.

5-Pregnene-3β,16α,20α-triol. As seen in Table 1, the TMS ether derivative of the urinary steroid (compound 3, Fig. 1) had the same RRT values as the reference 5-pregnene-3β,16α,20α-triol tri-TMS ether. The mass spectra of these two steroids were identical, too (for reference spectrum, see [23]). The results obtained show that this urinary steroid is 5-pregnene-3β,16α,20α-triol.

5-Pregnene-3β,17α,20α-triol. The TMS ether derivative of this steroid (compound 4, Fig. 1) had RRT values (Table 1) and a mass spectrum identical with 5-pregnene-3β,17α,20α-triol di-TMS ether[24]. On the basis of these results, compound 4 was identified as 5-pregnene-3β,17α,20α-triol. This steroid was present in both the mono- and disulphate fractions of urinary steroids.

In the silicic acid fraction V, the following five C_{19} steroid triols were found:

5α-Androstane-3α,16α,17β-triol. The TMS ether derivative of this steroid (compound 5, Fig. 1) had the same RRT values as reference 5α-androstane-3α,16α,17β-triol tri-TMS ether (Table 1). The mass spectrum of the TMS ether derivative of compound 5 gave a molecular ion at m/e 524 and a base peak at m/e 191 and ions indicating loss of a fragment with 103 mass units (due to vicinal hydroxyl groups), i.e. a mass spectrum typical of the TMS ether derivative of androstane-3,16,17-triols[22]. The *trans* position of the hydroxyl groups in the D-ring was confirmed by the fact that compound 5 did not form an acetonide[15]. The orientation of the hydroxyl group at carbon 3 was ascertained by oxidation with 3α-hydroxysteroid dehydrogenase, which led to the formation of 16α,17β-dihydroxy-5α-androstan-3-one (RRT of the TMS ether derivative on QF-1 = 2·88, see ref. [21]). On the basis of these results, compound 5 was identified as 5α-androstane-3α,16α,17β-triol.

5-Androstene-3β,16β,17α-triol and 5α-androstane-3β,16β,17α-triol. As seen in Fig. 1, the peak with the RRT values of 0·79 and 1·02 on QF-1 and SE-30 columns, respectively, seems to be quite symmetrical. However, GC-MS analyses revealed that this peak is formed by two steroids (compounds 6 and 7). As seen in Fig. 2, the main steroid in this peak had a mass spectrum typical of 5-androstene-3,16,17-triol tri-TMS ethers[21] and the minor one a mass spectrum identical with that of androstane-3,16,17-triol tri-TMS ethers (ions at m/e 524, 509, 434, 344 and 191, see ref. [22]). After hydrogenation of the silicic acid fraction V, the RRT values of this peak were the same as before hydrogenation and

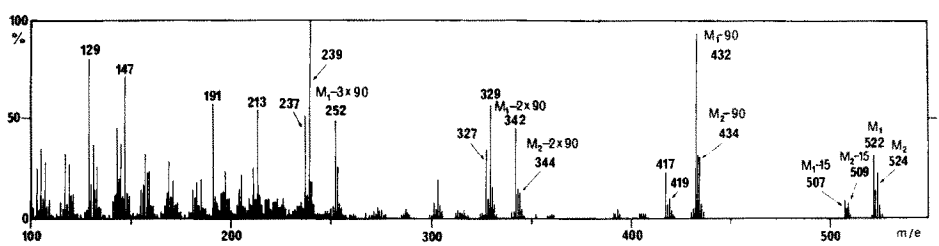

Fig. 2. Mass spectrum of the peak formed by the TMS ether derivatives of compounds 6 and 7 (5-androstene-3β,16β,17α-triol and 5α-androstane-3β,16β,17α-triol).

Table 1. Relative retention times (5α-cholestane = 1·00) of the derivatives of the C_{19} and C_{21} steroid triols identified in the present study and of reference compounds. 5α-Cholestane time: QF-1, 10–11 min; SE-30, 26–29 min. For conditions during GLC, see Fig. 1

	3% QF-1				2·2% SE-30			
	Reference steroid		Urinary steroid		Reference steroid		Urinary steroid	
Compound	TMS	Ac-TMS*	TMS	Ac-TMS	TMS	Ac-TMS	TMS	Ac-TMS
5α-Androstane-3α,16α,17β-triol	0·72	–	0·72	–	0·93	–	0·92	–
5α-Androstane-3β,16α,17β-triol	1·03	–	1·02‡	–	1·22	–	1·19†	–
5α-Androstane-3β,16β,17α-triol	0·80	–	0·79†	–	1·03	–	1·02†	–
5α-Androstane-3β,16β,17β-triol	1·14	1·09	1·10†	1·10	1·25	0·81	1·25†	0·79†
5-Androstene-3β,16α,17β-triol	0·97	–	0·97	–	1·20	–	1·19†	–
5-Androstene-3β,16β,17α-triol	0·79	–	0·79†	–	1·02	–	1·02†	–
5-Androstene-3β,16β,17β-triol	1·10	0·98	1·10†	0·98	1·25	0·79	1·25†	0·79†
5-Pregnene-3β,16α,20α-triol	1·45	–	1·43	–	1·80	–	1·81	–
5-Pregnene-3β,17α,20α-triol	2·22	1·22	2·22	1·23	1·74	1·15	1·74	1·18

*Acetonide trimethyl silyl ether derivative.
†Mixture of compounds.
‡In the latter part of the peak.

GC-MS analysis gave a mass spectrum identical with that of 5α-androstane-3β,16β,17α-triol tri-TMS ether. Neither compound 6 nor compound 7 was capable of forming an acetonide, which confirmed the *trans* position of the hydroxyl groups in the D-ring[15]. After oxidation with 3α-hydroxysteroid dehydrogenase, neither compound was changed, which showed the 3β-orientation of the hydroxyl group at carbon 3. The RRT values of the TMS ether derivatives of reference 5-androstene-3β,16β,17α-triol and 5α-androstane-3β,16β,17α-triol were the same as those of the TMS ethers of compounds 6 and 7, respectively (Table 1). The results obtained show that these urinary steroids were 5-androstene-3β,16β,17α-triol and 5α-androstane-3β,16β,17α-triol.

5-Androstene-3β,16α,17β-triol and 5α-androstane-3β,16α,17β-triol. These two steroids (compounds 8 and 9) were not separated on an SE-30 liquid phase, but their partial separation could be achieved on a QF-1 column (Fig. 1, Table 1). GC-MS analysis on a QF-1 column revealed that the former steroid was a 5-androstene-3,16,17-triol and the latter an androstane-3,16,17-triol[21, 22]. The RRT values of the TMS ether derivatives of compounds 8 and 9 were identical with the same derivative of reference 5-androstene-3β,16α,17β-triol and 5α-androstane-3β,16α,17β-triol, respectively (Table 1). Neither of these compounds was capable of forming an acetonide nor were they oxidizable with 3α-hydroxysteroid dehydrogenase. These results show the *trans* position of the hydroxyl groups in the D-ring and the 3β-orientation of the hydroxyl group at carbon 3. On the basis of these studies, compounds 8 and 9 were identified as 5-androstene-3β,16α,17β-triol and 5α-androstane-3β,16α,17β-triol, respectively. Both of these steroids were present as monosulphates and, in addition, small amounts of 5-androstene-3β,16α,17β-triol were found as a disulphate in the urine of some subjects.

In conclusion, the following C_{19} and C_{21} triol monosulphates were identified in normal female and male urine: 5α-androstane-3α,16α,17β-triol, 5α-androstane-3β,16α,17β-triol, 5α-androstane-3β,16β,17α-triol, 5α-androstane-3β,16β,17β-triol, 5-androstene-3β,16α,17β-triol, 5-androstene-3β,16β,17α-triol, 5-androstene-3β,16β,17β-triol, 5-pregnene-3β,16α,20α-triol and 5-pregnene-3β,17α,20α-triol. Only 5-pregnene-3β,17α,20α-triol and small amounts of 5-androstene-3β,16α,17β-triol were found as disulphates.

Quantitative aspects. In Table 2 the daily excretion of the monosulphates of the steroids characterised are given for 5 males and 3 females. The values are not corrected for methodological losses. On the liquid phases used during GLC, it was not possible to measure separately 5-androstene-3β,16α,17β-triol and 5α-androstane-3β,16α,17β-triol, 5-androstene-3β,16β,17α-triol and 5α-androstane-3β,16β,17α-triol as well as 5-androstene-3β,16β,17β-triol and 5α-androstane-3β,16β,17β-triol. However, GC-MS analyses showed that 80–90 per cent of these "mixture peaks" were formed by the Δ^5-unsaturated steroids. Recoveries of unconjugated 5-androstene-3β,16α,17α-triol, 5α-androstane-3α,16α,17β-triol and 5-pregnene-3β,17α,20α-triol added to urine samples after Sephadex LH-20 chromatography and carried through the different steps of the procedure were 70–80 per cent. As seen in Table 2, the main compounds among the steroids analysed were 5-androstene-3β,16α,17β-triol, 5-androstene-3β,16β,17α-triol and 5-pregnene-3β,17α,20α-triol. The ratio between the mean concentrations of 5-androstene-3β,16α,17β-triol, 5-androstene-3β,16β,17α-triol and 5-androstene-3β,16β,17β-triol was about 10:2:0·6 in female and 10:4:0·7 in male urine.

Table 2. Daily excretion of the monosulphates of C_{19} and C_{21} steroid triols by young subjects (aged 20–30 years). The values are expressed as μg of the free steroid/24 h

Steroid	Females (3 subjects) Mean	(Range)	Males (5 subjects) Mean	(Range)
5α-androstane-3α,16α,17β-triol	8	(5–13)	15	(5–35)
5-androstene-3β,16α,17β-triol and 5α-androstane-3β,16α,17β-triol*	424	(61–790)	599	(458–745)
5-androstene-3β,16β,17α-triol and 5α-androstane-3β,16β,17α-triol*	91	(26–124)	238	(159–352)
5-androstene-3β,16β,17β-triol and 5α-androstane-3β,16β,17β-triol*	24	(15–31)	44	(29–70)
5-pregnene-3β,16α,20α-triol	13	(< 5–27)	24	(< 5–54)
5-pregnene-3β,17α,20α-triol	52	(11–84)	174	(52–354)

*See text.

DISCUSSION

In the present investigation, three epimeric 5-androstene-3,16,17-triols were characterised and measured in normal female and male urine. Previously, 5-androstene-3β,16α,17β-triol and 5-androstene-3β,16β,17β-triol have been identified in normal adult urine as well as in urine of patients with endocrinologic disorders[3, 5, 25, 26]. In addition, 5-androstene-3β,16α,17β-triol has been shown to be a quantitatively important steroid in infant urine[27]. The third 5-androstene-3,16,17-triol identified, 5-androstene-3β,16β,17α-triol, has not so far been found in adult urine, but is present in human bile[21], in cord blood, meconium, faeces and urine of infants[28]. Of the four androstane-3,16,17-triols identified in the present study, only 5α-androstane-3α,16α,17β-triol has previously been shown to be present in human urine[6]. The two C_{21} steroid triols found as sulphates in urine have earlier been identified in human urine (see e.g. [7]).

From the quantitative point of view, 5-androstene-3β,16α,17β-triol and 5-androstene-3β,16β,17α-triol were the main compounds among the C_{19} triol sulphates in adult urine. Much greater amounts of unsaturated than saturated C_{19} steroid triols were present in urine, and this is in agreement with the ratio of unsaturated to saturated 3,16-dihydroxylated ketonic C_{19} steroids in adult urine[8]. It is of interest to observe that the quantitatively most important steroids have hydroxyl groups in the *trans* position in the D-ring. The same has been observed in the case of the epimeric oestriols (see e.g. [29]). This suggests that the *trans* position of the hydroxyl groups at carbons 16 and 17 is favored in the metabolism of C_{18} and C_{19} steroids.

Normal human urine contains several 16- or 17-hydroxylated C_{19} steroids which can be regarded as possible precursors of the steroid triols identified in the present study[8]. 16α-Hydroxydehydroepiandrosterone and 3β,17β-dihydroxy-5-androsten-16-one may be direct precursors of 5-androstene-3β,16α,17β-triol and the latter a precursor of 5-androstene-3β,16β,17β-triol, too. After administration of 16α-hydroxydehydroepiandrosterone or 16α-hydroxydehydroepiandrosterone sulphate, 5-androstene-3β,16α,17β-triol or its sulphate, respectively, were identified in urine[30, 31]. Accordingly, 16β-hydroxydehydroepiandrosterone can be assumed to be a precursor of 5-androstene-3β,16β,17α-triol and 5-androstene-3β,16β,17β-triol. 5-Androstene-3,17-diols and androstane-3,17-diols are

also potential precursors of 5-androstene-3,16,17-triols and androstane-3,16,17-triols, respectively. It has been shown that, at least in foetal liver, preferential 16α-hydroxylation of 5-androstene-3β,17β-diol rather than 5-androstene-3β,17α-diol occurs[32]. Human placenta, liver and intestine are able to epimerise 16α-hydroxyoestrone to its 16β-isomer[33], but this epimerisation has not so far been shown to occur in the metabolism of neutral steroids. It remains to be established whether the precursors of saturated C_{19} steroid triols are the corresponding unsaturated steroids or whether these triols are formed by reduction of the corresponding saturated dihydroxy monoketo C_{19} steroids. The presence of a number of saturated 3,16-dihydroxylated 17-ketonic C_{19} steroid sulphates in human urine[8] supports the latter alternative. YoungLai and Solomon[10, 11] have shown that 16α-hydroxydehydroepiandrosterone can be metabolised to 5α-androstane-3α,16α,17β-triol, but whether the metabolism proceeds *via* 16α-hydroxyandrosterone or 5-androstene-3α,16α,17β-triol is not known.

Almost all the steroids identified in this study were present in adult human urine as monosulphates. This is in contrast to most of the C_{19} steroid triols present in conjugated form in human bile, where these steroids mainly exist as disulphates[21].

It is of interest to observe that qualitatively and quantitatively the pattern of C_{19} steroid triols seems to be very similar in female and male urine. This might indicate that these potential precursors of oestrogens are not involved in the formation of oestrogens in non-pregnant females.

Analyses of urine samples collected at different trimesters of pregnancy are in progress. Preliminary results show that considerable amounts of 5-androstene-3,16,17-triols and androstane-3,16,17-triols are excreted in maternal urine during pregnancy. The same C_{19} steroid triol sulphates as in normal subjects as well as some other isomers are present in pregnancy urine. In addition, pregnancy urine contains some C_{19} steroids with four oxygen functions. The identification of these steroids will be the subject of a later communication.

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