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# PRESENCE OF 21-HYDROXYLATED NEUTRAL STEROID DISULPHATES IN TERM AMNIOTIC FLUID

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## SUMMARY

Gas–liquid chromatography and gas chromatography–mass spectrometry have been used in studies of neutral steroid sulphates in amniotic fluid collected at term. In addition to the compounds previously identified from this biological fluid, the neutral steroid disulphate fraction was found to contain 5-androstene-3$\beta$,16$\beta$,17$\alpha$-triol, 5$\alpha$-pregnane-3$\alpha$,20$\alpha$-diol, 3$\alpha$,21-dihydroxy-5$\alpha$-pregnan-20-one, 3$\beta$,21-dihydroxy-5-pregnen-20-one, 5$\alpha$-pregnane-3$\alpha$,20$\alpha$, 21-triol and 5-pregnene-3$\beta$,20$\alpha$,21-triol. The main compounds in the amniotic fluid had a 3$\beta$-hydroxy-$\Delta^5$ structure. Of the 21-hydroxylated steroids, the ratio of the concentrations of ketonic to non-ketonic compounds was about 10:1.

## INTRODUCTION

IT WAS shown by Villee *et al.* that human foetal adrenals have a high 21-hydroxylating activity[1, 2] and several C21-yl sulphates of 4-pregnene derivatives have recently been identified in umbilical cord blood[3, 4]. Also, a number of ketonic and non-ketonic 21-hydroxylated neutral steroid disulphates are present in late-pregnancy urine[5, 6].

In previous studies from this laboratory, several sulphate-conjugated neutral steroids were identified and quantitated in amniotic fluid collected at term[7] or during early and mid-pregnancy[8]. In this study, a search was made for the presence of 21-hydroxylated neutral steroid sulphates, possibly closely reflecting the activities of the foetal adrenal cortex, in pooled amniotic fluid collected at term.

## MATERIAL AND METHODS

Amniotic fluid was collected as described previously[7] at term of pregnancy and stored frozen until analysed.

All the reagents used were twice redistilled. The reference compounds were purchased from Ikapharm (Ramat-Gan, Israel) or given by Prof. W. Klyne (Steroid Reference Collection, London, England). Some reference compounds were prepared from available reference steroids by reduction with sodium borohydride (see[6]).

*The isolation* of a disulphate fraction of neutral steroids in amniotic fluid has been described in detail previously[7]. After solvolysis, the liberated steroids were fractionated on a 3 g column of silicic acid[9], and the fractions eluted with 20 ml of 27% ethyl acetate in benzene and of 33% ethyl acetate in benzene (fractions 2 and 3) were combined and a portion was subjected to thin-layer chromatography. The fractions eluted with ethyl acetate and methanol (fractions 4 and 5) were combined and analysed without further purification.

*Thin-layer chromatography* (TLC) of steroids was performed using precoated abrasion-resistant $F_{254}$-plates (Merck AG, No. 5715, 0·25 mm) and a solvent system cyclohexane/ethyl acetate (1/1, by vol., five developments). A zone 10·0–11·5 cm from the starting line was scraped off and eluted with methanol. After derivative formation, the steroids were analysed by gas–liquid chromatography and gas chromatography–mass spectrometry.

*Gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC–MS)* were carried out as described previously[10], using trimethyl silyl (TMS) ether, O-methyl oxime trimethyl silyl (MO-TMS) and O-isopropylidene trimethyl silyl (acetonide-TMS) ether derivatives.

*Oxidation* of steroids with chromium trioxide in acetone was done according to Sjövall and Sjövall [11].

*In quantitative analyses* a known amount of stigmasterol was added as internal standard and calculations were made as described previously[10].

## RESULTS

Figure 1 shows GLC analyses of the combined silicic acid fractions 2 + 3 and 4 + 5. In addition to the compounds previously identified in the disulphate fraction of term amniotic fluid (5-androstene-3$\beta$,17$\alpha$-diol = peak 1; 5-androstene-3$\beta$, 17$\beta$-diol = 2; 5-pregnene-3$\beta$,20$\alpha$-diol = 4; 5$\alpha$-pregnane-3$\beta$,20$\alpha$-diol = 5; 16$\alpha$-hydroxydehydroepiandrosterone* = 8; 16$\beta$-hydroxydehydroepiandrosterone = 9; 3$\beta$,17$\beta$-dihydroxy-5-androsten-16-one = 10; 5-pregnene-3$\beta$, 17$\alpha$, 20$\alpha$-triol = 12), some other steroids were present. One of these (compound 3, Fig. 1) gave a mass spectrum of the type given by pregnane-3, 20-diol[12]. As shown in Table 1, the relative retention times (RRT, 5$\alpha$-cholestane = 1·00) of the TMS ether derivatives of the compound in amniotic fluid were the same as those of the reference 5$\alpha$-pregnane-3$\alpha$,20$\alpha$-diol di-TMS ether. On the liquid phases used during GLC, however, 5$\alpha$-pregnane-3$\alpha$, 20$\alpha$-diol and 5$\beta$-pregnane-3$\beta$, 20$\alpha$-diol di-TMS ethers are not separated from each other (for RRT values, see e.g. [12]). After oxidation with chromium trioxide, only 5$\alpha$-pregnane-3, 20-dione was found by GLC and GC–MS analyses (for reference spectrum see ref. [5]), which confirmed the identification of compound 3 as 5$\alpha$-pregnane-3$\alpha$, 20$\alpha$-diol.

Compound 11 as a TMS derivative gave a mass spectrum typical of 5-androstene-3,16,17-triol tri-TMS ether[13–15]. Its RRT values (Table 1) were identical with those of 5-androstene-3$\beta$,16$\beta$,17$\alpha$-triol tri-TMS ether, different from those of the other epimers of the TMS derivatives of 5-androstene-3,16,17-triols[13]. In addition, compound 11 did not form an acetonide. Therefore, it was identified as 5-androstene-3$\beta$,16$\beta$,17$\alpha$-triol.

All the other steroids identified in this study displayed mass spectra typical of 21-hydroxylated $C_{21}$ steroids[5, 6, 11]. The criteria for their identification were the following:

*3$\alpha$, 21-Dihydroxy-5$\alpha$-pregnan-20-one.* The mass spectrum of the TMS ether derivative of compound 6 gave a molecular ion at m/e 478 and an intense base peak at m/e 257 as well as ions indicating the loss of a fragment with 103 mass units, i.e. a spectrum typical of 21-hydroxypregnanolone di-TMS ethers[5]. The RRT values of the steroid in amniotic fluid, and of reference 3$\alpha$, 21-dihydroxy-5$\alpha$-pregnan-20-one were identical both as TMS and MO-TMS ether derivatives (Table 1). As shown previously, 3$\alpha$, 21-dihydroxy-5$\alpha$-pregnan-20-one TMS ether is not completely separated from the corresponding 5$\beta$-isomer on QF-1 and SE-30 liquid phases[5], but by the TLC in the solvent system cyclohexane/ethyl acetate (1/1, v/v) these steroids are well separated. On the TLC plate the 21-hydroxypregnanolone from the amniotic fluid had the same mobility (10·5 cm from the

**Trivial and systematic nomenclature of steroids. Dehydroepiandrosterone*: 3$\beta$-hydroxy-5-androsten-17-one; *16$\alpha$-hydroxydehydroepiandrosterone*: 3$\beta$,16$\alpha$-dihydroxy-5-androsten-17-one; *16$\beta$-hydroxydehydroepiandrosterone*: 3$\beta$,16$\beta$-dihydroxy-5-androsten-17-one: *21-hydroxypregnenolone*: 3$\beta$,21-dihydroxy-5-pregnen-20-one: *corticosterone*: 11$\beta$, 21-dihydroxy-4-pregnene-3,20-dione: *stigmasterol*: (24*R*)-24-ethylcholesta-5, 22-dien-3$\beta$-ol.

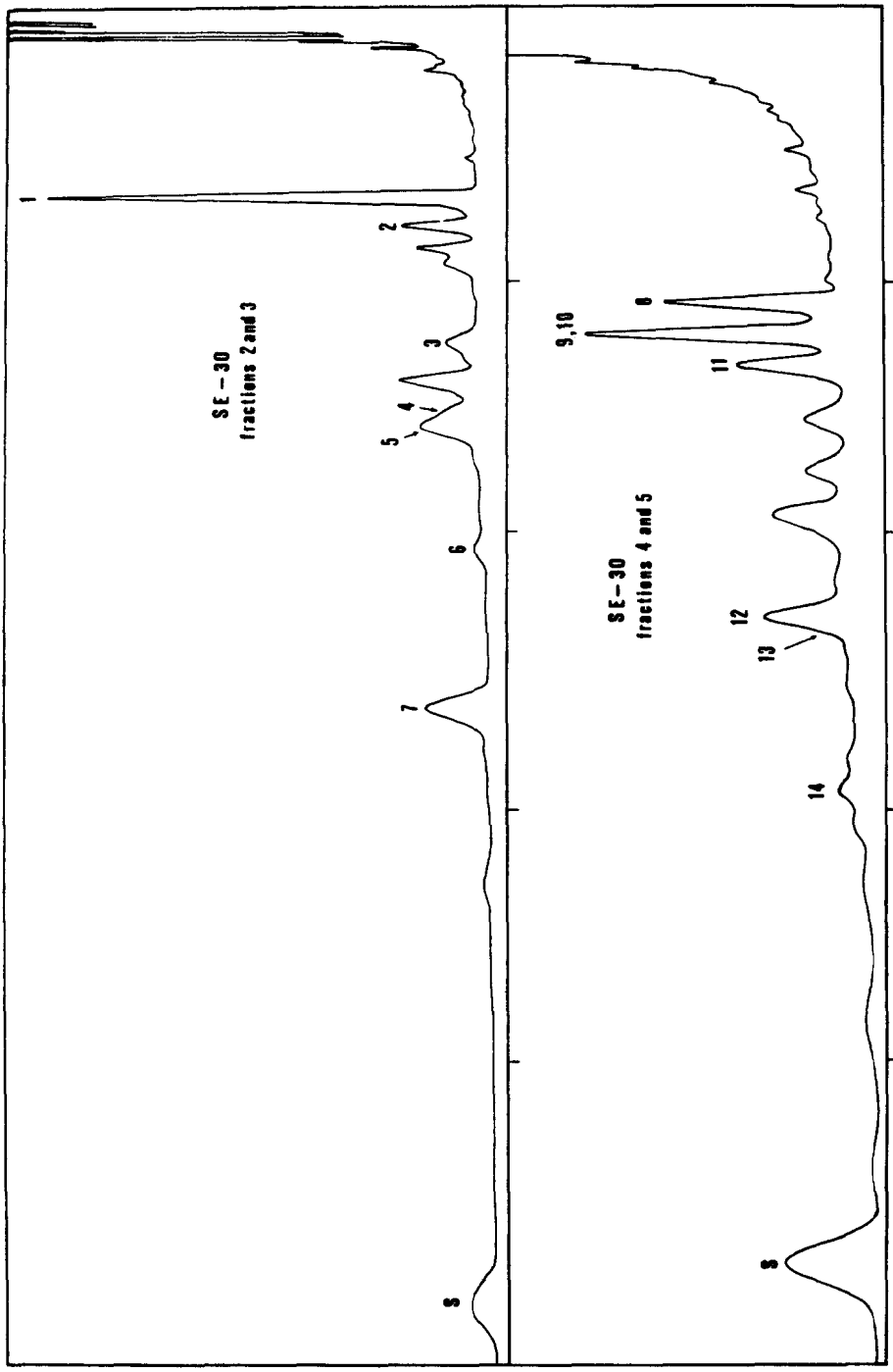


Fig. 1. Gas chromatographic analyses of the TMS ethers of steroids in the disulphate fraction of pooled amniotic fluid collected at term. For conditions, see Table 1. Peak identifications: see Tables 1 and 2 and text (S = stigmasterol internal standard).

Table 1. Relative retention times (5α-cholestane = 1·00) of the derivatives of steroids in the disulphate fraction of term amniotic fluid and of relevant reference compounds*. 5α-Cholestane time: QF-1, 9–11 min; SE-30, 26–29 min

| | | 3% QF-1 | | | | | | 2·2% SE-30 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Steroid in amniotic fluid | | | Reference steroid | | | Steroid in amniotic fluid | | | Reference steroid | | |
| No. | Compound Identification | TMS | MO-TMS | AC-TMS† | TMS | MO-TMS | AC-TMS | TMS | MO-TMS | AC-TMS | TMS | MO-TMS | AC-TMS |
| 3 | 5α-Pregnane-3α,20α-diol | 0·85 | — | — | 0·86 | — | — | 0·93 | — | — | 0·93 | — | — |
| 6 | 3α,21-Dihydroxy-5α-pregnan-20-one | 2·52 | 1·11 | — | 2·51 | 1·12 | — | 1·50 | 1·26 | — | 1·48 | 1·30 | — |
| 7 | 21-Hydroxypregnenolone | 3·24 | 1·40 | — | 3·24 | 1·44 | — | 1·92 | 1·80 | — | 1·90 | 1·83 | — |
| 11 | 5-Androstene-3β,16β,17α-triol | 0·79 | — | — | 0·80 | — | — | 1·02 | — | — | 1·03 | — | — |
| 13 | 5α-Pregnane-3α,20α,21-triol | 1·45‡ | — | 1·44‡ | 1·40 | — | 1·40 | 1·70§ | — | 1·17‡ | 1·82 | — | 1·19 |
| 14 | 5-Pregnene-3β,20α,21-triol | 1·85‡ | — | 1·77‡ | 1·85 | — | 1·77 | 2·27 | — | 1·45 | 2·25 | — | 1·45 |

Columns and conditions: 3% QF-1, 2 m × 3·5 mm, 215°C; 2·2% SE-30, 2 m × 3·5 mm, 225°C.

*For the RRT values of compounds 1, 2, 4, 5, 8, 9, 10 and 12, see ref. [7].

†Acetonide trimethyl silyl ether derivative.

‡Mixture of compounds.

§In the latter part of the peak.

starting line) as reference 3α,21-dihydroxy-5α-pregnan-20-one. The 5α-structure was further strengthened by oxidation with chromium trioxide, which led to the formation of 5α-androstane-3,17-dione and 5α-androstane-17-carboxylic acid [5]. The data thus obtained confirmed the presence of 3α,21-dihydroxy-5α-pregnan-20-one in term amniotic fluid.

*21-Hydroxypregnenolone.* The mass spectrum of the TMS ether of compound 7 gave a molecular ion at m/e 476 and a base peak at m/e 255 (M-(131 + 90)) as well as other fragments seen in the spectrum of the TMS ether of reference 21-hydroxypregnenolone (for reference spectrum, see [5]). The RRT values of the TMS and MO-TMS ether derivatives of the steroid in amniotic fluid and of the corresponding derivatives of reference 21-hydroxypregnenolone were identical, too (Table 1). On the basis of these results, this steroid in amniotic fluid was identified as 21-hydroxypregnenolone.

*5α-Pregnane-3α,20α,21-triol.* GC-MS analysis revealed that the latter part of the peak formed by the TMS ether derivative of 5-pregnene-3β,17α,20α-triol on the SE-30 column gave a mass spectrum typical of pregnane-3,20,21-triol tri-TMS ethers[11]. On the QF-1 column, this compound was eluted together with 16α-hydroxydehydroepiandrosterone. Owing to the small amounts of this steroid in the pools of amniotic fluid analysed, no attempt was made to purify it further. As shown in Table 1, the RRT values of the TMS and acetonide-TMS ether derivatives of compound 13 and of reference 5α-pregnane-3α,20α,21-triol were identical, which confirmed the identification of this steroid as 5α-pregnane-3α,20α,21-triol.

*5-Pregnene-3β,20α,21-triol.* The TMS ether derivative of this steroid (compound 14, Fig. 1) had a mass spectrum with a prominent base-peak at m/e 267 (M-(2 × 90 + 103)) and a molecular ion at m/e 550. The RRT values of the TMS and acetonide-TMS ethers (Table 1), as well as the mass spectra of these derivatives of compound 14 and of reference 5-pregnene-3β,20α,21-triol, were the same (for reference spectrum, see [16]); therefore, compound 14 was identified as 5-pregnene-3β,20α,21-triol.

*Quantitations.* Table 2 gives the concentrations of neutral steroid disulphates in human term amniotic fluid. 5-Pregnene-3β,20α-diol, 5α-pregnane-3β,20α-diol, 16β-hydroxydehydroepiandrosterone and 3β,17β-dihydroxy-5-androsten-16-one were determined on a QF-1 column, whereas the other compounds were quantitated with an SE-30 column.

Of the steroids identified in this study, 21-hydroxypregnenolone was quantitatively the most important (Table 2), being exceeded only by 5-androstene-3β, 17α-diol, identified previously[7]. The steroids devoid of a 3β-hydroxy-$\Delta^5$ structure were present in rather low concentrations.

## DISCUSSION

The steroids identified were present as disulphates and, as reported earlier [7], only small amounts of 16α-hydroxydehydroepiandrosterone were found in the monosulphate fraction. The presence of neutral steroid glucuronides in the amniotic fluid has not yet been investigated.

All the 21-hydroxylated steroid disulphates identified (3α,21-dihydroxy-5α-pregnan-20-one, 21-hydroxypregnenolone, 5α-pregnane-3α,20α,21-triol and 5-pregnene-3β,20α,21-triol) have previously been found in the disulphate fraction of late-pregnancy urine[5, 6]. In addition, 21-hydroxypregnenolone disulphate

Table 2. Concentrations of neutral steroids in the disulphate fraction of a pool of normal human amniotic fluid collected at term. The values are expressed as μg of the free steroid in 1000 ml of amniotic fluid and are uncorrected for methodological losses

| Compound | Identification | Concentration |
|---|---|---|
| 1 | 5-Androstene-3β,17α-diol | 236 |
| 2 | 5-Androstene-3β,17β-diol | 56 |
| 3 | 5α-Pregnane-3α,20α-diol | 36 |
| 4 | 5-Pregnene-3β,20α-diol | 29 |
| 5 | 5α-Pregnane-3β,20α-diol | 49 |
| 6 | 3α,21-Dihydroxy-5α-pregnan-20-one | 18 |
| 7 | 21-Hydroxypregnenolone | 122 |
| 8 | 16α-Hydroxydehydroepiandrosterone | 46 |
| 9 | 16β-Hydroxydehydroepiandrosterone | 5 |
| 10 | 3β,17β-Dihydroxy-5-androsten-16-one | 62 |
| 11 | 5-Androstene-3β,16β,17α-triol | 38 |
| 12 | 5-Pregnene-3β,17α,20α-triol | 36 |
| 13 | 5α-Pregnane-3α,20α,21-triol | Trace |
| 14 | 5-Pregnene-3β,20α,21-triol | 12 |
| | Total | 745 |

has been found in the urine of new-born infants[17] and of non-pregnant adult subjects[5].

The presence of 21-hydroxylated neutral steroids in amniotic fluid probably reflects the high 21-hydroxylating activity of the foetal adrenals[1, 2]. Compounds of this structure are transported across the placenta and in the case of corticosterone 21-sulphate it has been demonstrated that the transfer does not necessitate hydrolysis of the C21-yl sulphate[18]. Of the compounds with a C21 hydroxyl group in amniotic fluid, those with a 3β-hydroxy-$\Delta^5$ structure predominate, whereas in maternal urine saturated compounds are more abundant [5, 6]. The same has been observed in the case of 21-hydroxylated neutral steroids in pregnancy plasma[11] and in faeces[19]. Therefore, the large amounts of 21-hydroxylated neutral steroids in maternal urine are partly formed in the foetal compartment (introduction of a hydroxyl group at C21) and further subjected to reductive reactions in maternal tissues. Partly, they obviously are maternal metabolites of progesterone, because administration of large amounts of progesterone to a non-pregnant subject led to the appearance of C21-hydroxylated metabolites in the urine[20]. But it should be noted that saturated 21-hydroxylated neutral steroids are present, although in rather low concentrations, in amniotic fluid, as shown in this investigation. It cannot be stated at the present moment whether these compounds are metabolites of progesterone or of a precursor with a 3β-hydroxy-$\Delta^5$ structure. It has been shown that progesterone is converted to 21-hydroxylated metabolites in the foetal compartment[21, 22]. On the other hand, the foetal adrenal shows 3β-hydroxysteroid dehydrogenase activity for a limited number of steroids with a 3β-hydroxy-$\Delta^5$ structure[23–25]. Towards the end of pregnancy, the previously high activity of 3β-hydroxysteroid dehydrogenase in the foetal testes is considerably decreased[26] and therefore the possibility that the foetal testes contribute to the formation of the saturated 21-hydroxylated neutral steroids in amniotic fluid can be neglected.

Another clearcut difference in the composition of the 21-hydroxylated neutral steroid fraction in amniotic fluid and maternal urine at the end of pregnancy is that the ratio between non-ketonic and ketonic 21-hydroxylated steroids is higher in maternal urine[6] than in amniotic fluid. Therefore it is possible that reduction of the 20-keto group takes place primarily in the maternal compartment, although an enzyme system capable of reducing a 20-keto group is already present in the foetal liver during early and mid-pregnancy[27, 28].

In this investigation, the concentrations of the compounds previously determined in term amniotic fluid[7] were of the same magnitude as before. The concentration found for 3$\beta$,17$\beta$-dihydroxy-5-androsten-16-one was, however, larger as compared to those of the 16$\alpha$- and 16$\beta$-hydroxylated metabolites of dehydroepiandrosterone.

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