Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 101–105. Pergamon Press. Printed in Great Britain

STEROID GLUCURONIDES IN AMNIOTIC FLUID AT TERM

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(*Received* 12 *July* 1974)

SUMMARY

The steroid content of the glucuronide fraction isolated from term amniotic fluid by chromatography on Sephadex LH-20 was analysed by gas–liquid chromatography and gas chromatography–mass spectrometry. The following steroids were identified: estriol, 5-androstene-3β,16β,17α-triol, 3α-hydroxy-5β-pregnan-20-one, 5β-pregnane-3α,20α-diol, 3α,16α-dihydroxy-5α-pregnan-20-one, 3β,16α-dihydroxy-5β-pregnan-20-one, 3α,6α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3β,16α,20α-triol, and 5β-pregnane-3α,17α,20α-triol.

There was a striking difference in the qualitative composition of the neutral steroids in the glucuronide fraction and those in the steroid sulfate fraction from amniotic fluid. Most of the neutral steroid sulfates in amniotic fluid appear to have a 3β-hydroxy-5-ene structure, whereas the glucuronide fraction contains almost exclusively saturated C_{21} steroids, mainly metabolites of progesterone.

The neutral steroid glucuronide concentration was 945 μg/l, indicating that they are quantitatively the most important steroid conjugates so far investigated in term amniotic fluid.

The concentrations of unconjugated and conjugated estriol were of the same magnitude as reported by other investigators.

INTRODUCTION

It is generally accepted that fetal urine makes an important contribution to the steroid composition in term amniotic fluid. Thus it is probable that analysis of the steroid content of liquor amnii may provide valuable information on fetal steroid excretion under normal and pathological conditions.

Amniotic fluid has been found to contain only small amounts of unconjugated steroids [1–3]. In addition several conjugated neutral steroids [4–8] and estrogen conjugates [see 2, 5, 8–10] have been identified. However the mode of conjugation of the neutral steroids in amniotic fluid has not been fully established. The neutral steroid mono- and disulfates have been analyzed [6, 11, 12] and only small amounts of monosulfates detected [11] in contrast to fetal plasma, where these conjugates predominate [13].

The object of the present investigation was to determinate the qualitative and quantitative steroid glucuronide composition of amniotic fluid and to compare it with the other conjugated steroid fractions.

MATERIAL AND METHODS

Amniotic fluid was obtained by rupturing the membranes of 15 women, 38–41 weeks pregnant, at delivery, after uncomplicated pregnancies. The samples were pooled and stored at −18°C until analyzed.

All the reagents used were redistilled. The reference compounds: 5-androstene-3β,16β,17α-triol was obtained by reduction of 16β-hydroxydehydroepiandrosterone* with sodium borohydride. 3β,16α-Dihydroxy-5β-pregnan-20-one was kindly given to us by Prof. W. Klyne (Steroid Reference Collection, London, England), 3α,16α-dihydroxy-5α-pregnan-20-one and 3α,6α-dihydroxy-5β-pregnan-20-one were kindly supplied by Dr. O. Jänne, Helsinki, Finland, androsterone- and etiocholanolone glucuronides were supplied by Dr. J. F. Becker, [7α-^{3}H]-dehydroepiandrosterone glucuronide (specific activity 2,5 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, Mass.), radioactively labelled estriol-3-sulfate-16-glucuronide was kindly given to us by Dr. M. J. Tikkanen, Helsinki, Finland. Other reference compounds were purchased from Ikapharm (Ramat–Gan, Israel).

The method used for isolating the steroid glucuronides from amniotic fluid was essentially the same as described earlier for bile [14]. The procedure was as follows: (1) amniotic fluid was extracted with 10 vol. of acetone–ethanol (1:1, v/v); (2) the fractions of unconjugated steroids, steroid glucuronides and steroid mono- and disulfates were separated by chromatographing the extract on Sephadex LH-20 (see [14]); (3) unconjugated steroids were analyzed as described by Laatikainen *et al.*[13]; (4) the steroid glucuronides were hydrolyzed with β-glucuronidase (Ketodase, Warner–Chilcott, Morris Plains, N.J.). The reaction mixture, containing 600 Fishman units of the enzyme/ml, was incubated for 36 h at pH 5·0. Because estriol glucuronide was found to overlap with the monosulphate fraction on Sephadex LH-20, it was necessary to perform a β-glucuronidase hydrolysis on that fraction

* Trivial and systematic nomenclature of steroids. Androsterone: 3α-hydroxy-5α-androstan-17-one, etiocholanolone: 3α-hydroxy-5β-androstan-17-one, dehydroepiandrosterone: 3β-hydroxy-5-androsten-17-one, 16β-hydroxydehydroepiandrosterone: 3β,16β-dihydroxy-5-androsten-17-one, 16α-hydroxydehydroepiandrosterone: 3β,16α-dihydroxy-5-androsten-17-one, progesterone: 4-pregnene-3,20-dione, cortisol: 11β,17,21-trihydroxy-4-pregnene-3,20-dione,estriol: 1,3,5(10)-estratriene-3,16α,17β-triol, stigmasterol: (24R)-24-ethylcholesta-5,22-dien-3β-ol.

Table 1. Relative retention times (RRT) and concentrations (μg/of the free steroid/l ± S.D., nine determinations) of steroid glucuronides identified in amniotic fluid pool at term. Chromatographic conditions: 3% QF-1, 3% SE-30.5α-cholestane = 1·00

Compound	Identification	QF-1 215 C TMS A.f.	QF-1 215 C TMS Ref.	QF-1 215 C MO TMS A.f.	QF-1 215 C MO TMS Ref.	SE-30 225 C TMS A.f.	SE-30 225 C TMS Ref.	SE-30 225 C MO TMS A.f.	SE-30 225 C MO TMS Ref.	μg/l	a	b
1	3α-Hydroxy-5β-pregnan-20-one	1·61	1·60	0·87	0·87	0·65	0·65	0·82	0·82	43 ± 8	QF-1	II
2	5β-Pregnane-3α,20α-diol	0·91	0·92	--	-	0·96	0·95	—		281 ± 34	SE-30	III
3	5-Androstene-3β,16β,17α-triol	0·79	0·79	--	—	1·00*	0·97	—	--	trace		IV
4	3α,16α-Dihydroxy-5α-pregnan-20-one	1·60*	1·60	1·00	1·00	1·00*	1·02	1·24	1·24	223 ± 35	QF-1	IV
5	3β,16α-Dihydroxy-5β-pregnan-20-one	1·60*	1·58	0·99	1·00	1·00*	1·00	1·18	1·19			
6	3α,6α-Dihydroxy-5β-pregnan-20-one	2·00	2·01	1·08	1·08	0·92	0·94	1·18	1·18	166 ± 28	SE-30	IV
7	3ξ,6ξ-Dihydroxy-5ξ-pregnan-20-one	2·70	-	--		1·24	-		—	n.q.		IV
8	5α-Pregnane-3β,16α,20α-triol	1·38*	1·39	-		1·78*	1·78			87 ± 27	QF-1	IV
9	3ξ,16ξ,X-Trihydroxy-5ξ-pregnan-20-one	2·31	—	-		1·78*	—	—		n.q.		IV
10	5β-Pregnane-3α,17α,20α-triol	1·88	1·90	—	—	1·44	1·44	--		145 ± 24	SE-30	IV
11	Estriol	1·38*	1·38*		-	1·30	1·30			621 ± 48	SE-30	IV

A.f.—compound from amniotic fluid.
Ref. – reference compound.
a—Column used for quantitation.
b—Silicic acid fraction in which the steroid was eluted; 2 ml of 2% ethyl acetate in toluene (fraction I), 4 ml of 6% ethyl acetate in toluene (II), 4 ml of 18% ethyl acetate in toluene (III), and 4 ml of methanol (IV).
+—The quantitation is performed by subtracting the concentration of estriol on SE-30 from the concentration of estriol + compound 8 on QF-1.
* —Mixture of compounds.
n.q. – Not quantitated.

also. The liberated neutral steroids and estriol were extracted with ethyl acetate. The extracts were combined and the steroids were fractionated on a 200 mg silicic acid column (see [13] and Table 1); (5) the fractions of steroid mono- and disulfates were analyzed separately as described earlier by Luukkainen *et al.* [11[; (6) labelled estriol-3-sulfate-16-glucuronide was eluted in the same fraction as the steroid disulfates, and therefore enzymatic hydrolysis with *Helix pomatia* [15] was carried out on this fraction after the disulfates had been liberated by solvolysis. The liberated estriol was extracted with ethyl acetate and purified on a 200 mg silicic acid column [13].

The steroids were identified and quantified as their trimethylsilyl (TMS) or O-methyloxime trimethylsilyl (MO–TMS) ethers by gas–liquid chromatography (g.l.c.) and computer linked gas chromatography–mass spectrometry (GC–MS) (the programs for the computer were made by Mr. E. Soini). 3% SE-30 and 3% QF-1 liquid phases were used.

Thin layer chromatography (t.l.c.) was carried out on precoated abrasion-resistant F-254-plates (Merck Ab. No. 5717, 0·25 mm) in a solvent system containing cyclohexane–ethyl acetate–ethanol (62:38:1, by vol. five developments).

Steroids were reduced with 1 mg sodium borohydride in 1 ml methanol overnight.

Oxidation with CrO_3 in pyridine was performed as described by Gustafsson *et al.*[16].

For quantitation of the steroids by g.l.c. a known amount (10 μg) of internal standard (stigmasterol) was added to each sample before g.l.c. and peak areas compared as reported earlier [13].

RESULTS

Detection of steroids in amniotic fluid

In Table 1 the steroid glucuronides found in amniotic fluid are listed. The criteria on which the identification of the various compounds in the glucuronide fraction is based were the following:

Compounds 1 and 2 were identified as 3α-hydroxy-5β-pregnan-20-one (Compound 1) and 5β-pregnane-3α,20α-diol (Compound 2) as described by Eriksson *et al.*[17] and by Laatikainen *et al.*[18].

Figure 1 shows the g.l.c. traces of the steroid glucuronides eluted with methanol (fraction IV) from a 200 mg silicic acid column (see 13 and Table 1) after hydrolysis with Ketodase. The compounds were identified as follows:

Compound 3. The mass spectrum of this compound as its TMS ether was typical of a 5-androstene-3,16,17-triol [19]. The RRT values of compound 3 as a TMS ether were identical with the TMS derivative of 5-androstene-3β,16β,17α-triol, which can be separated from the other three 5-androstene-3β,16,17-triol isomers on the liquid phases used [20].

Compounds 4 and 5 and reference 3α,16α-dihydroxy-5α-pregnan-20-one and 3β,16α-dihydroxy-5β-pregnan-20-one had identical RRT values. They cannot, however, be separated on the liquid phases used. By t.l.c. (for details see Material and Methods) these compounds could be separated (8·1 cm and 5·5 cm from the starting point, respectively). When the amniotic fluid sample was subjected to t.l.c. two bands were found with mobilities identical to the reference compounds. The mass spectra of compounds 4 and 5 in

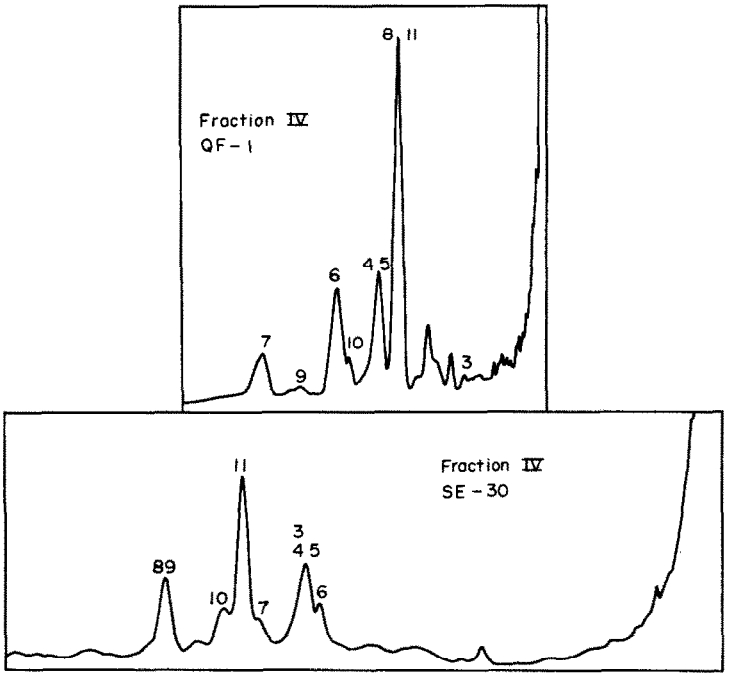

Fig. 1. Steroid glucuronides in amniotic fluid: gas–chromatographic analyses of TMS ethers of steroids in silicic acid fraction IV. For peak identifications, see Table 1 and text. Chromatographic conditions: 3% QF-1, 215°, 3% SE-30 temperature programmed 1°C/min from 200 to 240°C. 5α-Cholestane was eluted in 8 min on QF-1 and in 31 min on SE-30.

these two t.l.c. fractions were also typical of 3,16-dihydroxy-5α/β-pregnan-20-ones [21].

Compound 6 had a mass spectrum identical with that of 3α,6α-dihydroxy-5β-pregnan-20-one-TMS ether [22]. The RRT values were also the same as those of the reference compound.

Compound 7 was tentatively identified as 3ξ,6ξ-dihydroxy-5ξ-pregnan-20-one on the basis of its mass spectral fragmentation, which was very similar to that of the TMS ether of 3α,6α-dihydroxy-5β-pregnan-20-one [22]. Because no reference 6-hydroxylated pregnanolones other than 3α,6α-dihydroxy-5β-pregnan-20-one were available definite identification of this steroid was not possible.

Compound 8, as its TMS ether, gave a mass spectrum typical of a pregnane-3,16,20-triol [23]. Its RRT values were also the same as reference 5α-pregnane-3β,16α,20α-triol. The 5α configuration was confirmed by oxidizing the compound with CrO_3 in pyridine. The product was identical to 16α-hydroxy-5α-pregnan-3,20-dione (see [16]).

Another compound with a mass spectrum similar to pregnane-3,16,20-triol TMS ether was found but due to its low concentration, further characterization was not possible.

Compound 9. The TMS derivative of this compound gave a mass spectrum similar to one reported by Shackleton *et al.*[22] who tentatively identified the compound as 3ξ,16ξ,X-trihydroxy-5ξ-pregnan-20-one.

Compound 10. The mass spectra and RRT values of the TMS derivative of this compound were identical to those of reference 5β-pregnane-3α,17α,20α-triol [23].

Compound 11 was identified as estriol according to the criteria of Adlercreutz and Luukkainen[24].

Quantitation of steroids in amniotic fluid

For evaluating the precision of the method used, nine quantitations of steroid glucuronides in the amniotic fluid pool were carried out. The values obtained were not corrected for methodological losses. The recoveries for androsterone- and etiocholanolone glucuronides and for tritiated dehydroepiandrosterone glucuronide added to the amniotic fluid pool varied from 65–80%. The quantitative values for the steroid glucuronides are shown in Table 1. 3α,16α-Dihydroxy-5α-pregnan-20-one and 3β,16α-dihydroxy-5β-pregnan-20-one could not be separated as their TMS ethers by g.l.c. and were therefore quantitated together. Because 5α-pregnane-3β,16α,20α-triol could not be separated from estriol on QF-1 and from compound 9 on SE-30, it was quantitated by subtracting the concentration of estriol on SE-30 from the compound peak of estriol and 5α-pregnane-3β,16α,20αtriol on QF-1. All the neutral steroid glucuronides except 5-androstene-3β,16β,17α-triol quantitated in this study were saturated C_{21} steroids, of which 5β-pregnane-3α,20α-diol was quantitatively the most important.

Estriol and progesterone were the only unconjugated steroids detected in the amniotic fluid analyzed. Both compounds were identified on the basis of their g.l.c. and GC–MS properties [24, 25], and were quantitated as reported earlier [13]. The concentrations of estriol and progesterone found were 26 ± 4 μg/l and 25 ± 4 μg/l respectively, (mean ± S.D., 12 determinations).

Fractions of mono- and disulfates were analyzed essentially as described by Luukkainen *et al.*[11] and Jänne and Vihko[6]. Qualitatively the same steroid sulfates reported by these authors were found by the method used.

Estriol sulfoglucuronide, which was eluted in the same fraction as the disulfates on Sephadex LH-20 (see Material and Methods) had a concentration of 128 ± 27 μg/l (mean ± S.D., eight determinations).

DISCUSSION

The fetus is capable of forming glucuronide conjugates of estriol and pregnanediol [26, 27], although it is generally considerated that the ability is rather limited, but increases with advancing gestational age [28]. Fetal bile also contains steroid glucuronides [29]. It is probable that at least part of the steroid glucuronides detected in amniotic fluid are of fetal origin.

The total amount of steroid glucuronides in amniotic fluid at term was 1566 μg/l. The main compound present was estriol glucuronide. The concentration of steroid sulfates in amniotic fluid [6, 10, 11] was calculated to be about 1000 μg/l. Thus quantitatively, the steroid glucuronides are the most important steroid conjugates in amniotic fluid at term so far investigated. From the qualitative point of view it is noteworthy that in the glucuronide fraction, 5-androstene-3β,16β,17α-triol was the only 3β-hydroxy-5-ene steroid. The neutral steroid glucuronides in amniotic fluid were almost exclusively saturated C_{21} steroids, probably metabolites of progesterone. In contrast, the main neutral steroids found in the sulfate fraction have a 3β-hydroxy-5-ene structure [6, 11]. These steroids are also known to occur as sulfates in the fetal circulation [13].

The main neutral steroid in the glucuronide fraction was 5β-pregnane-3α,20α-diol. It was present at a concentration of 281 μg/l, slightly higher than that reported by Schindler and Siiteri[4]. 3α,6α-Dihydroxy-5β-pregnan-20-one was identified in the glucuronide fraction and evidence for the presence of another 6-hydroxylated pregnanolone was also obtained. Previously a number of 6β-hydroxylated cortisol derivatives have been identified in amniotic fluid by Lambert and Pennington[1]. The origin of the 6-hydroxylated C_{21} steroids in amniotic fluid is obscure, because in addition to the placenta and the fetal organism (see [30]), the nonpregnant adult [31] is also able to produce these compounds. 6-Hydroxylation has been found to be a quantitatively important step in cortisol metabolism in newborn infants [32], and considerable amounts of 3α,6α-dihydroxy-5β-pregnan-20-one has been found in the urine of the newborn by Shackleton *et al.*[22]. In addition, the excretion of 6-oxygenated metabolites of progesterone in maternal urine increases steadily throughout pregnancy [33]. Therefore it is probable that the 3α,6α-dihydroxy-5β-pregnan-20-one in amniotic fluid originates mainly from the feto-placental unit.

Three 16-hydroxylated C_{21} steroids, namely 3α,16α-dihydroxy-5α-pregnan-20-one, 3β,16α-dihydroxy-5β-pregnan-20-one and 5α-pregnane-3β,16α,20α-triol were identified and quantified in this study. These compounds, apparently products of fetoplacental progesterone metabolism, also occur as glucuronides in fetal bile [29]. The concentration of 16-hydroxylated neutral steroids in the glucuronide fraction was about twice that of the 6-hydroxylated pregnanolones. Bird *et al.*[34] have shown that 16-hydroxylation of C_{21} steroids is a more efficient process in the fetus than hydroxylation at position 6.

The mode of conjugation of the 16-hydroxylated 3β-hydroxy-5-ene steroids quantitated in amniotic fluid by Schindler and Siiteri [4] has not yet been determinated in full. They found the mean concentration of 16α-hydroxydehydroepiandrosterone in amniotic fluid to be 797·7 μg/l. Only a part of these 3β-hydroxy-5-ene steroids exists as mono- and disulfates [6, 11]. These steroids were not found in the glucuronide fraction in this study and therefore further investigations are necessary to elucidate the mode of conjugation of the remainder of these steroids.

The concentrations of unconjugated estriol and progesterone (26 μg/l and 25 μg/l respectively) found in this study are in reasonable agreement with earlier reports [2, 3]. The concentration of estriol in amniotic fluid at different stages of pregnancy has been investigated by several authors (see [2, 5, 8–10, 35]). The amounts of estriol glucuronide and estriol sulfoglucuronide determinated in the present study are of the same order of magnitude as reported by others.

Same steroids identified and quantitated in this study have also been found in the urine of newborn infants [22], suggesting that an important source of the steroids in amniotic fluid is fetal urine. It has also been reported that the concentration of unconjugated estriol and of the different estriol conjugates in amniotic fluid and in fetal urine at term is quite similar [35].

The factors regulating the steroid composition of liquor amnii during pregnancy are not known. It is, however, obvious that the mechanism of inflow and outflow change during the course of pregnancy and that they are different for unconjugated steroids and for the various steroid conjugates. By analyzing the steroid content, especially in the different conjugated fractions, of amniotic fluid, it is possible to obtain valuable information on steroid metabolism in the fetoplacental unit. In addition, pathological changes and hormonal disturbances in the fetoplacental unit could be expected to affect the steroid composition of amniotic fluid.

Acknowledgements—The skilful assistance of Miss Marjatta Vallas is gratefully acknowledged. This investigation was supported by a grant from the National Research Council for Medical Sciences, Finland and from the Jalmari and Rauha Ahokas Foundation. We are grateful to Prof. H. Adlercreutz for allowing us the use of the GC–MS instrument at the Department of Clinical Chemistry, University of Helsinki.

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