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DETERMINATION OF CONJUGATED STEROIDS IN AMNIOTIC FLUID

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SUMMARY

Steroid conjugates were extracted from amniotic fluid samples with the non-ionic resin Amberlite XAD-7. After a two-step hydrolysis procedure two fractions of steroids, glucuronides, and sulfates and sulfoglucuronides, were obtained. The quantitation of the steroids was performed by glass capillary gas chromatography. The following concentrations (μg/l) of steroids were found in the glucuronide fraction of 20 amniotic fluid samples in uncomplicated pregnancies at term: mean (range) pregnanediol 183 (41–369), pregnanolone 43 (12–81), 3α,6α-dihydroxy-5β-pregnan-20-one 48 (20–135), 3α,16α-dihydroxy-5α-pregnan-20-one 161 (50–391), pregnanetriol 60 (29–119), and estriol 616 (117–1713). Concentrations of steroids in the fraction containing sulfates and sulfoglucuronides were: 16α-hydroxypregnenolone 52 (20–165), 16α-hydroxyDHA 165 (14–781), 3β,17β-dihydroxy-5-androsten-16-one 53 (15–120), and estriol 218 (51–485). In two cases of anencephaly concentrations of pregnanediol and other progesterone metabolites were within the normal range but those of estriol precursors and estriol were low or undetectable.

INTRODUCTION

Amniocentesis is commonly used nowadays for determination of foetal lung maturity in the surveillance of high-risk pregnancies. It has been suggested that steroids in the amniotic fluid may reflect changes in the foetal condition as has already been found in the case of oestriol [1]. This subject, however, has been insufficiently studied.

The steroids in amniotic fluid are mainly in the conjugated form. In a previous study [2] we found that, quantitatively, steroid glucuronides predominated. Neutral steroid mono- and disulfates have also been determined [3, 4], and various conjugates of estriol in amniotic fluid have been studied [5, 6]. Steroid sulfates are transferred more rapidly through the foetal membranes than steroid glucuronides [7, 8] so the mode of conjugation influences the turnover of steroids in amniotic fluid. Therefore we considered it valuable to determine steroid glucuronides in amniotic fluid separately from those with the sulfate group.

We have used a simple method for extraction of steroid conjugates from amniotic fluid with a non-ionic resin Amberlite XAD-7. To obtain good specificity and sensitivity glass capillary gas chromatography was used for quantitation of the steroids.

* Trivial and systematic nomenclature of steroids: Dehydroepiandrosterone; 3β-hydroxy-5-androsten-17-one, 16α-hydroxyDHA: 3β,16α-dihydroxy-5-androsten-17-one, pregnanolone: 3α-hydroxy-5β-pregnan-20-one, pregnanediol: 5β-pregnane-3α-20α-diol, pregnanetriol: 5β-pregnane-3α,17α,20α-triol, 16α-hydroxypregnenolone: 3β,16α-dihydroxy-5-pregnen-20-one, stigmasterol: (24R)-24-ethylcholesta-5,22-dien-3β-ol.

MATERIAL AND METHODS

Amniotic fluid samples were obtained from 20 uncomplicated pregnancies at term either by rupturing the membranes through the amnioscope during delivery or at elective induction of labour at 38–41 weeks of gestation. Samples contaminated with blood or meconium were discarded. Fourteen male (mean weight 3900 g) and 6 female (mean weight 3643 g) babies, all healthy, were born to these mothers. The amniotic fluid samples were centrifuged at 3000 rev./min for 5 min and the supernatant stored at 18 C until analyzed.

The sources of reference compounds were as described previously [2]. Estriol-16-glucuronide* was kindly given to us by Prof. H. Adlercreutz, Helsinki. All solvents were redistilled. The non-ionic resin, Amberlite XAD-7, was purchased from Serva Ltd., Heidelberg, Germany. The resin was soaked in water, the fine particles removed by swirling and pouring away the supernatant. The resin was then washed with methanol, chloroform–methanol (1:1, V/V), methanol again and finally with water and kept under water until used [9].

Gas chromatography. Glass capillaries with an internal diameter of 0.35 mm were drawn from soda glass tubes using a vertically operating Shimadzu Type GDM-1 glass-drawing machine. For etching, glass capillaries were filled with HCl gas and sealed at both ends and placed in an oven at 350°C for 3 h [10]. After the etching procedure the columns were coated with 10% OV-225 liquid phase in dichlormethan using a dynamic method. A 7.5 m OV-225 column was installed in a Carlo Erba Model 2101 gas chromatograph equipped with a flame ionization detector.

Hydrogen was used as a carrier gas. The sample, dissolved in 2 μl of hexane, was injected without stream splitting using a direct injection technique described by Grob [11].

Procedure. 2.5 to 5 ml of amniotic fluid was diluted with water to a final volume of 9 ml and 1 g of Amberlite XAD-7 was added. After 60 min in a rotating mixer (30 rev./min) the resin was allowed to settle to the bottom of the tube and the supernatant removed with a Pasteur pipette. The resin was gently washed with 3 ml of water. Conjugated and unconjugated steroids were extracted from the resin with 2 × 6 ml of methanol. The methanol phase was evaporated and the sample dissolved in 5 ml of water. Unconjugated steroids were then extracted with 2 × 5 ml of diethyl ether–ethyl acetate (1:1, V/V). The combined ether ethyl acetate phase was backwashed with 1 ml of water. The combined water phase was submitted to β-glucuronidase hydrolysis [2] and the deconjugated steroids extracted with diethyl ether–ethyl acetate (1:1, V/V). The remaining steroid conjugates in the water phase were extracted with Amberlite XAD-7 and submitted to hydrolysis by *Helix Pomatia* extract [12].

The steroids were purified and fractionated using a 150-mg Hi-Flosil (Applied Science Lab., Inc.) silica gel column in toluene. Cholesterol and impurities were first eluted with 4 ml of 4% ethyl acetate in toluene (Fraction I). The steroids were then eluted in two fractions: Fraction II: by using 4 ml 20% acetate and Fraction III with 70% ethyl acetate in toluene.

A suitable amount (5–10 μg) of an internal standard, stigmasterol, was added to the steroid fractions and trimethylsilyl ethers of steroids were prepared. The steroids determined in this study have been identified in amniotic fluid earlier [2–4]. The compounds in the chromatograms (Fig. 1) were localized by repeating the chromatogram of the sample with a small amount of reference steroid. The steroids were quantitated by comparing their peak height responses to that of the internal standard. The differences in peak height responses were taken into account in these calculations by first obtaining the peak height responses of appropriate reference steroid and internal standard mixtures.

Table 1. Results of 8 analyses of conjugated steroids in an amniotic fluid pool. The values are expressed as μg/l of free steroid

Compound	Mean ± S.D.
Glucuronides	
Pregnanolone	60 ± 6.0
Pregnanediol	263 ± 35
3α,6α-Dihydroxy-5β-pregnan-20-one	52 ± 5.6
3α,16α-Dihydroxy-5α-pregnan-20-one	154 ± 13
5β-Pregnane-3α,17α,20α-triol	45 ± 4.8
Estriol	650 ± 21
Sulfates and sulfoglucuronides	
16α-HydroxyDHA	146 ± 15
3β-17β-Dihdroxy-5-androsten-16-one	46 ± 4.5
16α-Hydroxypregnenolone	46 ± 15
Estriol	163 ± 15

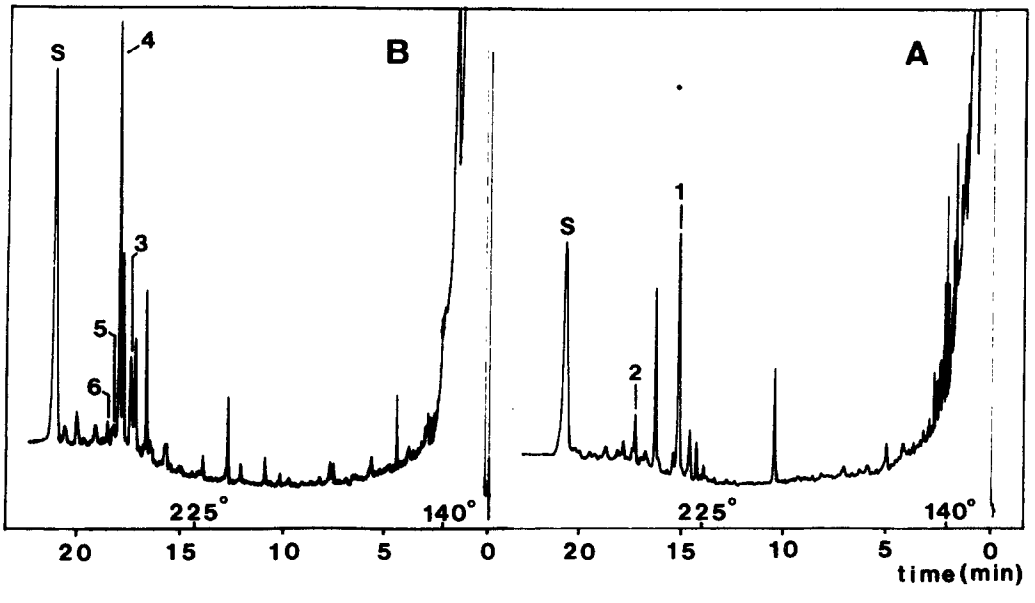

Fig. 1. Gas chromatographic analyses of the steroid TMS ethers in the glucuronide fraction from amniotic fluid. A: silica gel fraction II, B: fraction III. 1: pregnanediol, 2: pregnanolone, 3: 3α,16α-dihydroxy-5α-pregnan-20-one, 4: estriol, 5: pregnanetriol, 6: 3α,6α-dihydroxy-5β-pregnan-20-one. S: internal standard, stigmasterol. Chromatographic conditions: An 7.5-m OV-225 glass capillary column. 1/1000 of the sample in 2 μl of hexane was injected into the beginning of the column at room temperature, and the temperature was raised as shown in the figure.

Table 2. Concentrations of conjugated steroids in amniotic fluid samples in uncomplicated pregnancies at term and in anencephalic pregnancies. The values are expressed as μg/l and not corrected for methodological losses

	Glucuronides						Sulfates and sulfoglucuronides			
Case	Pregnanediol	Pregnanolone	3α.6α-dihydroxy-5β-pregnan-20-one	3α.16α-dihydroxy-5α-pregnan-20-one	Pregnanetriol	Estriol	16α-Hydroxy-dehydroepi-androsterone	3β.17β-Dihydroxy-5-androsten-16-one	Estriol	16α-Hydroxy-pregnenolone
Male foetuses										
1	261	60	56	274	50	853	80	70	232	59
2	216	51	39	116	58	934	781	59	485	165
3	50	23	32	84	119	1049	66	67	231	33
4	142	41	37	240	46	341	279	67	223	110
5	97	36	46	152	54	527	83	72	145	26
6	41	12	24	173	29	497	44	44	93	35
7	369	81	52	222	52	1713	135	57	253	23
8	134	55	61	111	54	900	245	120	331	51
9	75	22	20	59	39	216	14	19	51	24
10	135	47	37	140	65	647	485	74	403	128
11	196	44	81	277	60	480	82	57	134	42
12	97	31	135	146	90	479	130	39	221	58
13	232	42	82	391	75	402	168	42	282	60
14	125	32	25	65	37	117	23	15	54	23
Female foetuses										
15	69	18	24	50	89	239	50	62	89	27
16	376	74	56	245	84	902	52	48	148	25
17	274	58	40	114	47	497	258	52	361	49
18	359	41	40	196	53	491	181	32	317	53
19	304	70	37	108	55	378	28	23	99	20
20	116	24	27	65	53	660	123	39	217	38
Mean	183	43	48	161	60	616	165	53	218	52
± S.E.	24	4.3	6.0	20	4.8	82	41	5.3	27	8.7
Anencephaly										
21	140	30	54	92	25	33	N.D.*	15	30	15
22	160	37	36	60	23	55	N.D.	11	20	16

* N.D. = Not detectable.

RESULTS

The efficiency of the extraction step with Amberlite XAD-7 was determined by adding labeled unconjugated and conjugated steroids to amniotic fluid samples. Following recoveries were found: progesterone 88–97%, cortisol 92–68%, DHA glucuronide 79–94% and DHA sulfate 100–109%. The recoveries of estriol-16-glucuronide and DHA sulfate carried through the whole procedure varied from 81–100% and from 78 to 91%, respectively.

Figure 1 shows GLC analyses of amniotic fluid steroids in the glucuronide fraction. Using 2.5 to 5 ml samples the detection limit for steroid determinations was about 10 ng/ml. When a 5 ml sample of water was analyzed only a few impurity peaks above this limit were present in the chromatograms.

The precision of the method was evaluated by performing 8 repeat analyses on 5 ml samples from an amniotic fluid pool (Table 1). Concentrations of conjugated steroids determined in amniotic fluid samples at term can be seen in Table 2. The mean total estriol concentration was 834 μg/l, 73% being in the glucuronide fraction and 27% in the fraction containing sulfates and sulfo-glucuronides.

Results of steroid determinations in amniotic fluid samples in two pregnancies with anencephalic foetuses are seen in Table 2. In both cases the metabolites of progesterone, e.g. pregnanediol and pregnanolone were in the range found in uncomplicated pregnancies but the levels of estriol and estriol precursors were very low or undetectable.

DISCUSSION

A non-ionic resin, Amberlite XAD-7 has been used earlier for extraction of bile salts from serum [9]. In this study it was shown to extract efficiently both unconjugated and conjugated steroids from amniotic fluid. After hydrolysis of conjugates and purification of freed steroids using silica gel chromatography, sufficiently pure steroid fractions were obtained for quantitation using glass capillary gas chromatography. Compared with steroid quantitation using packed columns, which we have used in a previous study [2], the use of capillary columns gave increased specificity and sensitivity for quantitation of steroids in amniotic fluid. The methodological variation was also within reasonably narrow limits (Table 1).

The mean total concentration of conjugated estriol in amniotic fluid at term was 834 μg/l, which agrees with the findings of several other investigators (reviewed in 13). A considerable interindividual variation was found, the lowest value being 171 μg/l and the highest 1970 μg/l. Two major estriol conjugates, estriol-16-glucuronide and estriol-3-sulfate-16-glucuronide are known to represent up to about 70% of the total conjugated estriol in amniotic fluid at term [5, 6]. The mean ratio of estriol in the glucuronide fraction to that in the fraction of sulfates and sulfoglucuronides was 3.1 and ranged from 1.4 to 6.8 in the present study. This confirms the earlier findings [5, 6, 14] that the glucuronide conjugate of estriol predominates in amniotic fluid.

The present concentration of pregnanediol in amniotic fluid at term agree well with the earlier findings of Schindler and Ratanasopa [15] and Klopper [16]. 6α- And 16α-hydroxylated pregnane derivatives are also quantitatively important progesterone metabolites in amniotic fluid, their total amount exceeding that of pregnanediol (Table 2). The progesterone concentration in amniotic fluid declines steadily with advancing gestation [17], but little is known about progesterone metabolites in this respect.

The conjugate pattern of steroids in amniotic fluid differs from that in foetal blood [18]. The foetal kidneys retain steroid sulfates, e.g. DHA and 16α-hydroxyDHA sulfate which are important precursors of estrogen synthesis. On the other hand, steroid sulfates disappear rapidly from the amniotic cavity, because fetal membranes are able to hydrolyze steroid sulfates [7, 8]. As a result, glucuronides and also double conjugates [4, 6] accumulate in amniotic fluid.

The present results on steroid pattern in amniotic fluid in two anencephalic pregnancies confirm the earlier findings [15, 19]. Due to the hypoplastic foetal adrenal cortex the concentrations of oestriol precursors and estriol were low or undetectable. Concentrations of pregnanediol and other progesterone metabolites were, however, in the range found in normal pregnancies (Table 2).

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