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Cannulation in situ of equine umbilicus. Identification by gas chromatography-mass spectrometry (GC-MS) of differences in steroid content between arterial and venous supplies to and from the placental surface

D.E. Marshall[a], D.B. Gower[b], M. Silver[c], A. Fowden[c], E. Houghton[a,*]

[a]*Horseracing Forensic Laboratory Ltd, Newmarket Road, Fordham, Ely, Cambridgeshire, CB7 5WP, UK*
[b]*Drug Control Centre, King's College London, Manresa Road, London, SW3 6LX, UK*
[c]*Physiology Laboratory, University of Cambridge, Cambridge, CB2 3EG, UK*

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Abstract

Equine umbilicus was cannulated in utero and a series of cord plasma samples removed for analysis. After steroid extraction and derivatisation, gas chromatographic-mass spectrometric (GC-MS) analysis demonstrated large differences in steroid content between the plasma samples obtained from the umbilical artery and vein, the blood supplies leading to and from the placental surface, respectively. 3β-Hydroxy-5,7-androstadien-17-one, dehydroepiandrosterone, pregnenolone, 3β-hydroxy-5α-pregnan-20-one, 5-pregnene-3β,20β-diol and 5β-pregnane-3β,20β-diol were identified as major constituents in extracts from umbilical arterial plasma samples, mostly as unconjugated steroids. Together with 5α-pregnane-3,20-dione, these steroids were identified in extracts from umbilical venous plasma samples but at significantly reduced levels to those determined in arterial plasma samples. Oestradiol-17α, dihydroequilin-17α and dihydroequilenin-17α were identified in extracts (mostly sulphate-conjugated) from both umbilical arterial and venous plasma samples, much larger amounts being detected in the plasma sampled from, rather than to, the placental surface. Equilin, equilenin, oestrone, oestradiol-17β, dihydroequilin-17β and dihydroequilenin-17β were not detected in the present studies. Isomers of 5(10)-oestrene-3,17β-diol together with 5(10),7-oestradiene-3,17β-diol and its possible oxidative artifact, 5(10),7,9-oestratriene-3,17β-diol, were tentatively identified only in sulphate-conjugated extracts from umbilical venous plasma samples. No glucuronic acid-conjugated steroids could be detected. The implications of this work in the elucidation of the biosynthetic pathways leading to both the formation of oestrogens and C_{18} neutral steroids at the placental surface are discussed.

Keywords: 3β-Hydroxy-5,7-androstadien-17-one; Dehydroepiandrosterone; Isomeric 5(10)-oestrenediols; 5(10),7-oestradienediol; Ring-B unsaturated oestrogens; Equine; In situ cannulation; GC-MS

1. Introduction

By cannulating afferent and efferent blood supplies and measuring levels of steroids to and from an organ, an assessment of the steroidogenic processes and/or enzymic reactions occurring within the tissue may be made. Using such techniques and by measuring oestrogen content in plasma sampled from the uterine artery and vein, the placenta has been identified as the site of oestrogen formation during the later stages of equine pregnancy [1]. These in situ data complement the results obtained from earlier in vitro metabolic studies

Abbreviations: Testosterone, 17β-hydroxy-4-androsten-3-one; oestrenediol, 5(10)-oestrene-3,17α(β)-diol; dehydroepiandrosterone, DHA, 3β-hydroxy-5-androsten-17-one; Δ^7-DHA, 3β-hydroxy-5,7-androstadien-17-one; dihydroequilin-17α(β), 1,3,5(10),7-oestratetraene-3,17α-diol; dihydroequilenin-17α(β), 1,3,5(10),6,8-oestrapentaene-3,17α(β)-diol; oestradiol-17α(β), 1,3,5(10)-oestratriene-3,17α(β)-diol; oestrone, 3-hydroxy-1,3,5(10)oestratrien-17-one, pregnenolone, 3β-hydroxy-5-pregnen-20-one, 5α-pregnanedione, 5α-DHP, 5α-pregnane-3,20-dione, 5-pregnenediol, 5-pregnene-3β,20β-diol; pregnanediol, 5β-pregnane-3β,20β-diol; pregnanolone, 3β-hydroxy-5α-pregnan-20-one; GC-MS, Gas chromatography-mass spectrometry; OFN, oxygen free nitrogen; TIC, total ion chromatogram; TLC, thin layer chromatography.

* Corresponding author. Tel.: 01638-720500; fax: 01638-724200.

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using placental tissue preparations [2]. The in vitro studies undertaken in this Laboratory recently [3] confirmed the placenta as being the site of oestrogen formation in the pregnant mare but further delineated this activity to the chorion. Further it was demonstrated that oestradiol-17β was preferentially formed when testosterone was used as substrate and that the mechanism included the formation of carbonyl function at C-17 [3].

High concentrations of both classical oestrogens (oestrone, oestradiol-17α and -17β) together with the ring B-unsaturated oestrogens (equilin, equilenin, dihyroequilin-17α and -17β and dihydroequilenin-17α and -17β) are present in the blood and urine of mares in the second half of pregnancy. DHA has been shown to act as substrate for classical oestrogen formation both from in vivo and in vitro studies. Although the foetal adrenal cortex is clearly a source for DHA, the foetal gonads have been identified as the major source of DHA in the peripheral blood circulation in pregnant mares. It has also been identified as a major constituent in umbilical arterial plasma samples. A gonadal-placental link for DHA has been established in the pregnant mare [1,4,5]. A structurally related compound, 3β-hydroxy-5,7-androstadien-17-one (Δ^7-DHA) has been shown to act as a precursor to the ring B-unsaturated oestrogens in in vitro studies and, from cannulation studies, shown to be secreted from the gonad [6,7].

In this paper, gas chromatography-mass spectrometry (GC-MS) was used to identify the steroids present in extracts from equine umbilical cord plasma samples in attempts to throw light on the mechanism of formation of oestrogens and other steroids in the equine placental surface. A direct gonadal-placental link is now proposed for Δ^7-DHA.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all materials and chemicals were obtained from Sigma Chemical Co., Fancy Road., Poole, Dorset, UK, while HPLC grade solvents were obtained from Rathburn Chemicals Ltd., Walkerburn, Scotland. Kieselgel H-60 was purchased from BDH Chemical Co., Poole, UK. C_{18} Sep-Pak cartridges were purchased from Waters Chromatography Division, Millipore Corporation, Milford, MA, USA.

2.2. Steroids

Where possible, identification of steroids from plasma sample extracts was confirmed by comparison with authentic reference steroids, prepared as described below or obtained from either Sigma Chemical Co., or from Steraloids (UK) Ltd., 47 Station Road., New Barnet, Herts, UK. 3β-Hydroxy-5,7-androstadien-17-one (Δ^7-DHA), as the acetate, was prepared by the method of Antonucci et al. [14] and generously donated by Dr A. D. Tait. Before use in our studies, [16,16,17-$^2H_3$]-5α-androstane-3α,17β-diol was prepared and used as an internal marker at a concentration of 10 ng/μl in GC-MS [15].

2.3. Animals

All surgical procedures were performed at the Physiology Department, University of Cambridge. Four pony mares (ranging in gestational age 254–300 days as judged by last known covering dates and rectal palpation) were premedicated with acepromazine (0.4 mg/kg), anaesthesia being induced using sodium thiopentane (10 mg/kg). After intubation, anaesthesia was maintained with sodium pentabarbitone (70 mg/kg, intravenously). All mares were artificially ventilated using a mixture of O_2 and CO_2 to maintain maternal arterial blood gases within a normal range. The uterus was exposed using either a mid-line or half-supine incision and the umbilicus manipulated to the surface of the uterus. The vessels of the uterus were circumflexed to expose the main maternal uterine artery and vein. Catheters were inserted into arterial and venous branches of the uterus and umbilicus, such that the cannuli tips were advanced to lie in main areas of blood flowing to and from the placenta and foetus, respectively [16]. The four indwelling catheters were maintained in place by light suturing. Samples of blood from each cannulus (10 ml umbilical, 50 ml maternal supplies) were withdrawn into heparinised tubes at 30 min intervals over a 2–4 h period. Collection days, ranging from 254–300 days, were chosen since these reflect the hypertrophy and regression of the foetal gonads and the known changes in oestrogen content. Portions of the blood samples were used to measure blood gases, pH and packed cell volume in assessment of foetal and maternal well-being; all data fell within the range previously reported for conscious animals [17]. Blood samples were spun (1000 g_{av}, 10 min, 4°C) and the plasma aspirated for storage (−20°C) until required for analysis. Only data from cord plasma samples will be presented in the present communication.

2.4. Extraction of steroids

Analysis of samples was carried out at the Horseracing Forensic Laboratory. Initially, a complex extraction procedure, including the use of Sephadex-LH 20 chromatography, was adopted to isolate the

unconjugated, glucuronide- and sulphate-conjugated steroids from the foetal and arterial plasma samples [18]. However, by GC-MS analysis, the umbilical plasma sample extracts were shown to lack glucuronic acid-conjugated steroids, so that a simpler methodology could be adopted routinely.

The plasma sample (5 ml) was applied to an activated C_{18} Sep-Pak cartridge and the unconjugated steroids eluted using diethyl ether (10 ml). The eluant was dried (Na_2SO_4) and the ether was evaporated under oxygen-free nitrogen (OFN). The sulphate conjugates were then eluted from the C_{18} Sep-Pak cartridges using ethyl acetate:methanol (80:20 v/v, 10 ml), containing 4 drops of concentrated H_2SO_4. This sample was heated at 50°C for 2 h to hydrolyse the sulphate-conjugates, after which the acidified solution was washed sequentially with saturated solutions of $NaHCO_3$ and NaCl. The organic phase was then dried using Na_2SO_4 and evaporated to dryness under OFN. The residue was redissolved and small portions (1/20th) of the free and sulphate-conjugated extracts were derivatised as methyloxime-trimethyl silyl (MO-TMS) ethers for GC-MS analysis (see below). The residual amounts of the free steroid extracts from the umbilical arterial plasma samples were pooled and further purified using normal phase silica column chromatography (as below).

2.5. Normal phase column chromatography

Kieselgel silica H-60 (5 g in hexane) was slurry-packed into a glass column and sedimented using slight positive pressure. A layer of acid-washed sand was applied to the top of the column to minimise disturbance on sample application. The column was then equilibrated using 1,2-dichloroethane:ethanol (95:5, v/v). The pooled free steroid extract from umbilical arterial plasma samples (obtained as described above) was dissolved in 1,2-dichloroethane:ethanol (95:5, v/v, 50 μl). After application to the column, elution was carried out using the same solvent. Fractions (3 ml) were collected and subjected to spectrophotometric analysis. Fractions of interest were derivatised (MO-TMS ethers) for GC-MS analysis.

2.6. Spectrophotometric-ultraviolet (UV) analysis

The solvent was removed from fractions isolated from column chromatography and the residues dissolved in methanol (1 ml). UV spectra were generated over the range 200–350 nm in quartz cuvettes using either a SP800 or PU7800 UV/VIS series spectrophotometer (Philips Scientific, Cambridge, UK). For comparison, the UV spectrum of authentic reference material, 3β-hydroxy-5,7-androstadien-17-one (Δ^7-DHA), was also generated.

2.7. Derivatisation

Extracts for derivatisation were transferred to screw-capped vials and any residual solvent removed (OFN) prior to derivatisation. The dried residues were dissolved in a solution of methoxyamine hydrochloride in redistilled pyridine (2.5% w/v, 50 μl) and the reaction allowed to proceed at room temperature overnight. The pyridine was removed under a stream of OFN at 30°C and the methyloxime (MO)-residues silylated using *N*,*O*-bis-trimethylsilyl acetamide (BSA, 50 μl) and trimethylchlorosilane (TMCS, 25 μl). The trimethylsilyl (TMS) ether reaction was allowed to proceed at room temperature overnight, after which excess silylating reagents were removed under OFN at 30°C. It should be noted that the MO and TMS ether derivatisation steps may be performed at 60°C for 30 and 60 min, respectively. Residues were taken up in a small volume of chloroform:hexane (1:1, v/v), then passed through a short column of Sephadex LH-20 and eluted using chloroform:hexane (1:1v/v, 2 ml). After removal of the solvent under OFN, the MO-TMS ether derivatives were dissolved in undecane containing [16,16,17-$^2H_3$]-5α-androstane-3α,17β-diol (10 ng/μl) which acted as an internal standard for GC-MS analysis.

2.8. Gas chromatographic-mass spectrometric (GC-MS) analysis

GC-MS analysis was carried out on a Hewlett Packard 5890 series gas chromatograph using an OV-1 fused silica column (25 m × 0.25 mm id, phase 0.25 μ) with helium as carrier gas. The injection port temperature was maintained at 220°C and split/splitless injections were made. The oven temperature was initially maintained at 150°C for 2 min after sample injection and then temperature-programmed to increase at 20°C min^{-1} up to 220°C and finally at 5°C min^{-1} to 300°C. Final column temperature was maintained for 5 min. Thc GC was interfaced to a Finnigan Ion Trap detector (ITD) which was operated in the electron impact (EI) mode (ionising voltage 70 eV) over the scan range 100–550 amu.

For measurement of steroid concentrations, the ratio of a defined ion for each compound analysed to the ion, m/z 244 for the internal standard [16,16,17-$^2H_3$]androstanediol, was determined. This method provided an assessment of the relative amounts in the arterial and venous blood supplies.

3. Results

Sample preparation methods were originally used which allowed for the separation of free (unconju-

Table 1
Steroids identified in equine umbilical arterial and venous plasma samples[f]

Steroid	Fraction identified		Mol. wt.	GC-RRT		Major fragment ions (base peak is underlined)	
	Arterial	Venous		Isolated	Standard	Isolated	Standard
DHA	U[b]	U[b]	389	1.06	1.05	374(11); 358(37); 268(100); 129(70).	374(18); 358(49); 268(100); 129(60).
Δ^7-DHA	U	U	387	1.075	1.08	387(2); 297(8); 282(100); 256(27).	387 (8); 372(3); 297 (16); 282 (100); 256(32).
Pregnenolone	U	U	417	1.25	1.26	417(9); 402(30); 386(85); 312(35); 296(27); 239(51); 100(100).	417(20); 402(40); 386(95); 312(31); 296(20); 239(83); 100(100).
Pregnanolone[c]	U	U	420	1.26	1.26	404(7); 388(98); 298(6); 243(13); 100(100).	[a]
5-Pregnenediol[d]	U	U	462	1.27	1.28	462(4); 372(10); 282(6); 267(6); 117(100).	462(2); 372(6); 282(4); 267(4); 117(100).
Pregnanediol[e]	U	U	464	1.28	1.28	449(2); 347(4); 269(5); 117(100).	449(1); 347(2); 284(5); 269(5); 117(100).
Pregnanedione (5α-)	U	U	374	1.28	1.29	374(4); 343(100); 326(5); 288(47); 275(24); 244(5); 117(20).	374(19); 343(100); 288(64); 275(45); 244(23); 117(10).
DHEq-17α	U, S	S	414	1.1	1.1	414(47); 324(77); 309(100); 296(10); 282(8).	414(31); 324(67); 309(100); 282(13).
DHEq-17β	N.D.	N.D.	414	–	1.6	–	414(100); 324(12); 309(24); 283(30).
DHEqN-17α	U, S	S	412	1.15	1.15	412(14); 322(35); 307(100); 281(3).	412(13); 322(52); 307(100); 281(4).
DHEqN-17β	N.D.	N.D.	412	–	1.23	–	412(100); 322(21); 307(15); 281(56).
Oestradiol-17α	N.D.	S	416	1.07	1.07, 1.05	416(100); 326(22); 285(60); 232(14); 129(18).	416(100); 326(36); 285(89); 232(15); 129(19).
Oestradiol-17β	N.D.	N.D.	416	–	1.13, 1.11	–	416(100); 326(17); 285(49); 232(10); 129(17).
Oestrenediol	N.D.	S	420	1.00	0.98	330(100); 240(19); 225(7); 199(7); 129(29).	330(100); 240(26); 225(12); 199(12); 129(26).
Oestradiendiol	N.D.	S	418	0.99	[a]	418(5); 328(38); 313(8); 238(82); 223(100); 197(20).	[a]
Oestratriendiol	N.D.	S	416	1.07	[a]	416(2); 401(12); 326(58); 311(100); 236(33); 221(58); 195(13).	[a]
Oestrone	U, S	N.D.	371	1.06	1.08	Poor mass spectral data obtained	371(92); 340(110); 324(11); 282(15); 272(12); 231(11).

[a] authentic standards were not available.
[b] U, unconjugated (free); S, sulphate-conjugated; N.D., not detected;.
[c] 3β,5α;.
[d] 3β,20β;.
[e] 3β,5β,20β.
[f] Samples were extracted, purified and derivatised (as MO-TMS ethers or bis TMS ethers) as described in Materials and methods. Where possible identification was based on comparison with authentic reference steroids. Relative retention time compared with RT of [16,16,17-$^2H_3$] androstane-3α,17β-diol (internal standard), absolute RT 14.5 min. Steroid name abbreviations: DHEq-17α(β), dihydroequilin-17α(β); DHEqN-17α(β), dihydroequilenin-17α(β); oestrenediol, isomeric-5(10)-oestrene-3,17-diol; oestradienediol, 5(10),7-oestradiene-3,17-diol; oestratrienediol, 5(10),6,8-oestratriene-3,17-diol.

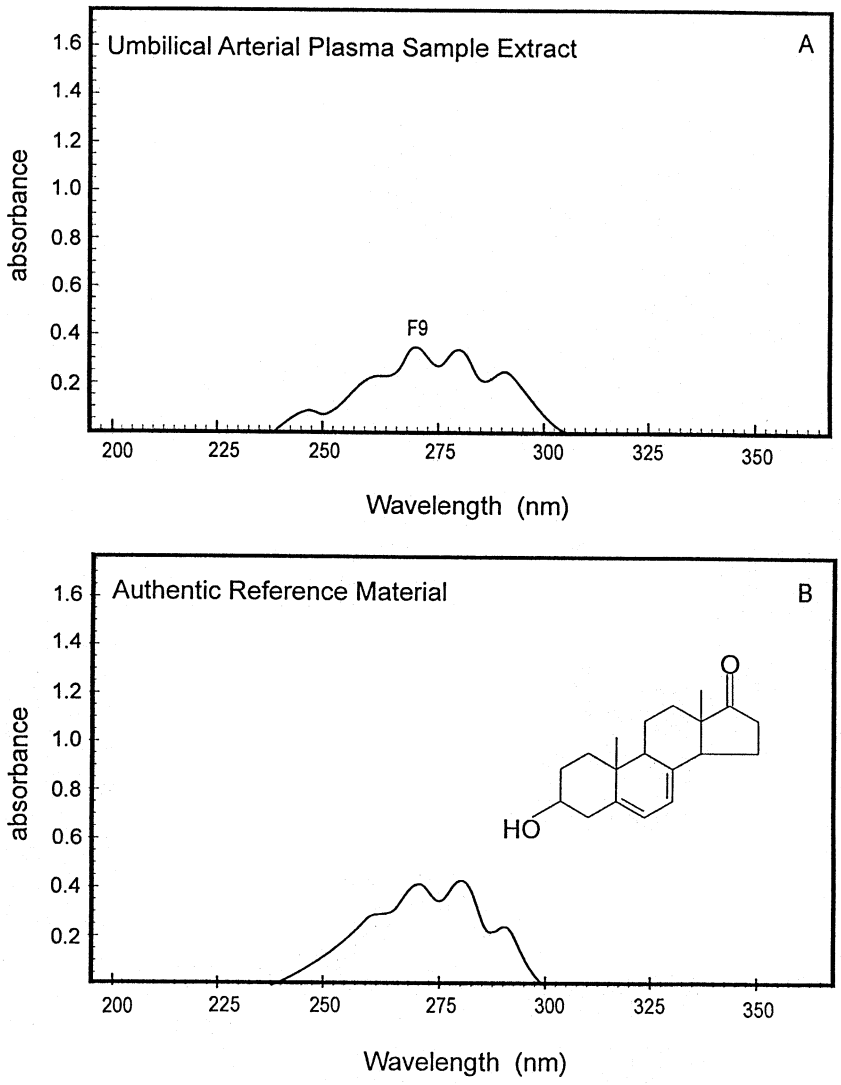

Fig. 1. UV analysis of equine umbilical arterial plasma extract. After extraction of plasma, the steroid-containing extract was subjected to column chromatography on Kieselgel H-60. Elution was achieved with 1,2-dichloroethane:ethanol(95:5, v/v) fractions (3 ml each) being collected. The UV spectrum generated is shown for fraction 9 (27–30 ml). For other details see Materials and methods.

gated), glucuronic acid- and sulphate-conjugated steroids. Using these methods and analysis of the resultant extracts by GC-MS, no glucuronic acid-conjugated steroids were detected in any extracts obtained from either umbilical arterial or venous plasma samples.

DHA was identified in the unconjugated extracts obtained from umbilical plasma samples as judged by its GC retention time (RT) and fragmentation pattern (M^+, m/z 389, Table 1). Another steroid was identified which, by mass spectrometry, displayed a molecular weight (MO-TMS ether) of M^+ m/z 387 and base peak of m/z 282 (Table 1). After purification using column chromatography, the UV pattern generated for fraction 9 eluted between 27 and 30 ml (see Materials and methods) indicated the presence of a distinctive triplet in the 272, 282, 292 nm region (Fig. 1). This was indicative of a conjugated bond system and was identical to that obtained for the authentic reference material, 3β-hydroxy-5,7-androstadien-17-one (Δ^7-DHA). Similarly, GC-MS analysis, in comparison to the authentic reference material indicated, a GC RT, molecular weight and fragmentation pattern identical to those for Δ^7-DHA (Table 1). No other C_{19} steroids were identified in these umbilical arterial plasma extracts (Table 1). Both DHA and Δ^7-DHA were found in all the unconjugated residues obtained from umbilical plasma samples but with no demonstrable presence in the sulphate-conjugated extracts (Table 2. In all four mares (gestational age 280–300 days) much lower concentrations of DHA and Δ^7-DHA were detected in venous as compared with arterial plasma samples. For 254 days gestation, however, interference in some chromatograms did not allow us to obtain quantitative data for DHA and Δ^7-DHA concen-

Table 2
Estimation of steroids present in the unconjugated and sulphate-conjugated fractions from equine umbilical arterial and venous plasma samples. – Means integration of peaks not possible owing to interference on chromatogram. ϕ, *syn*- and *anti*-forms

Steroid[a]	Ions used for SIM[b]	Gestational age (days) (number of analyses)															
		254 (2)				280 (4)				284 (3)				300 (3)			
		Arterial		Venous		Arterial		Venous		Arterial		Venous		Arterial		Venous	
		U[c]	S[c]	U	S	U	S	U	S	U	S	U	S	U	S	U	S
DHA	268	–	N.D.	0.10±0.03	N.D.	1.9±0.6	N.D.	0.03±0.05	N.D.	1.4±0.4	N.D.	0.03±0.06	N.D.	1.3±0.1	N.D.	N.D.	N.D.
Δ^7-DHA	282	–	N.D.	0.23±0.3	N.D.	8.4±3.0	N.D.	0.50±0.01	N.D.	5.4±0.7	N.D.	0.10±0.20	N.D.	4.4±0.6	N.D.	N.D.	N.D.
Pregnenolone	100	–	–	0.10±0.02	N.D.	3.3±0.7	N.D.	0.20±0.02	N.D.	4.8±1.2	0.03±0.06	0.50±0.10	N.D.	6.0±0.7	0.02±0.03	0.2±0.04	N.D.
Pregnanolone	100	–	–	0.27±0.02	N.D.	1.7±0.4	N.D.	0.30±0.05	N.D.	3.8±1.2	0.02±0.03	0.77±0.09	N.D.	4.8±1.3	0.01±0.02	0.57±0.1	N.D.
Pregnanedione	343	–	–	0.38 ϕ±0.01 0.4ϕ±0.01	N.D.	N.D.	N.D.	0.50ϕ±0.1 0.6ϕ±0.04	N.D.	N.D.	N.D.	1.1ϕ±0.19 1.2ϕ±0.14	N.D.	N.D.	N.D.	0.95ϕ±0.02 0.9ϕ±0.23	N.D.
5-Pregnenediol	117	–	–	0.05±0.02	N.D.	6.2±2.4	N.D.	0.14±0.02	0.03±0.06	11.9±2.7	0.30±0.2	0.50±0.1	0.1±0.03	17.7±1.0	0.16±0.14	0.2±0.02	N.D.
Pregnanediol	117	–	–	0.07±0.03	N.D.	2.7±0.6	N.D.	0.15±0.08	0.10±0.03	6.3±1.8	0.20±0.1	0.40±0.1	N.D.	4.3±1.6	0.1±0.10	0.2±0.05	N.D.
Oestradiol-17α	416	–	2.0±0.2	N.D.	2.7±1.0	0.1±0.1	0.6±1	N.D.	8.80±2.8	0.03±0.05	6.2±0.4	N.D.	6.3±0.5	0.1±0.1	0.4±0.4	N.D.	3.1±0.1
DHEq-17α	309	–	5.2±0.8	N.D.	7.0±3.2	0.3±0.1	0.1±2.2	N.D.	14.5±1.6	0.1±0.1	9.7±0.4	N.D.	12.2±1.6	0.1±0.1	0.6±0.7	N.D.	2.6±1.4
DHEqN-17α	307	–	4.4±1.3	N.D.	5.8±3.4	0.3±0.1	0.8±1.5	N.D.	12.3±4.5	0.1±0.1	9.6±0.8	N.D.	9.2±0.7	0.05±0.1	0.5±0.5	N.D.	3.6±0.4

[a] Steroid abbreviations are those given in legend to Table 1.

[b] An appropriate fragment ion was selected and the area of the peak generated from this was measured in relation to the area of the base peak of the internal standard [16,16,17-$^2H_3$]-androstane-3α,17β-diol, a known amount of which was added to the plasma extract for GC-MS analysis. The results represent the mean and S.D.

[c] U, unconjugated (free); S, sulphate-conjugated. N.D. means no peaks, i.e. no ion detected for integration.

trations in unconjugated fractions from arterial blood samples. We cannot, therefore, make comparisons as noted above for 280–300 days gestation but can state nevertheless that these two steroids were both present in venous (unconjugated) fractions, although undetectable in sulphate-fractions of both arterial and venous blood samples.

The C_{21} compounds, pregnenolone, pregnanolone, 5-pregnenediol, 5β-pregnanediol and pregnanedione were also identified in 'free' extracts from umbilical plasma samples (Tables 1 and 2) on the basis of GC RTs and mass spectral data. In the case of the last four named steroids, isomers could be resolved by GC analysis. For isomeric pregnanediols the RRTs were 1.30, 1.32 for 5α,3β,20β and 5α,3β,20α respectively and 1.28, 1.24 and 1.21 for 5β,3β,20β, 5β,3α,20α and 5β,3α,20β respectively. It was therefore possible to identify the isolated pregnanediol (RRT 1.28) as the 5β,3β,20β isomer. In the case of 5-pregnenediol, RRTs for the 3β, 20α and 3β, 20β isomers were 1.30 and 1.28 respectively. On this basis, it seems likely that the isolated material (RRT 1.27) was the 3β,20β isomer. For pregnanedione however, the authentic 5α-isomer was unavailable but the RRT for 5β-pregnanedione was 1.288. It seems likely therefore that the isolated material (RRT 1.277) was the 5α-isomer. For pregnanolone the authentic 3β, 5α-isomer was not available to us, whereas the 3β,5β compound had a RRT of 1.178, clearly distinct from that of the isolated material (RRT 1.26). We therefore infer that this should be assigned the 5α,3β- configuration.

As with DHA and Δ^7-DHA, much lower concentrations of these C_{21} compounds were detected in the corresponding extracts from venous plasma samples in all four mares studied (gestational ages 280–300 days). Again, no similar comparison was possible for 254 days. Although 5α-pregnanedione was absent from chromatograms generated for umbilical arterial plasma extracts, it was identified in the unconjugated extracts of venous plasma samples (Table 2). The C_{21} compounds were concentrated in the unconjugated steroid extracts obtained from umbilical arterial plasma samples (280–300 days gestation), with negligible demonstration of sulphate conjugation (Tables 1 and 2).

Oestrone was putatively identified using GC RT data and selected ion monitoring in the unconjugated and sulphate-conjugated plasma extracts from a few umbilical arterial plasma samples (Table 1), although only poor full scan mass spectral data were obtained (data not shown). No equilin, equilenin (17-oxo oestrogens) or 17β-reduced oestrogens were identified in the present study. However, oestradiol-17α (M^+, m/z 416), dihydroequilin-17α (M^+, m/z 414) and dihydroequilenin-17α (M^+, m/z 412) were all identified in extracts from both umbilical arterial and venous plasma samples on the basis of GC RTs and mass

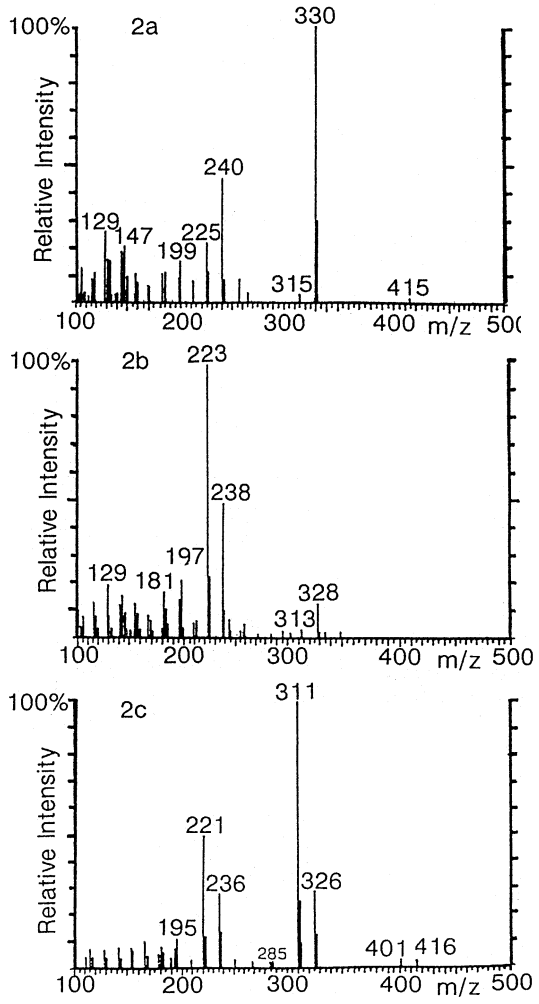

Fig. 2. Mass spectra of (a) 5(10)-oestrenediol, (b) 5(10),7-oestradienediol and (c) 5(10),7,9-oestratrienediol (as bis-TMS ethers), identified in extracts from equine umbilical venous plasma.

fragmentation patterns which agreed well with those for the appropriate authentic steroids. Although the presence of these oestrogens could be detected in both the free and sulphate-conjugated extracts of umbilical arterial plasma samples, all oestrogens appeared exclusive to the sulphate-conjugated extracts from umbilical venous plasma samples (Table 2). Moreover, in two of the four animals (gestational ages 280 and 300 days), a significant increase in the estimated levels of sulphated oestrogens in venous, relative to arterial, umbilical plasma samples was demonstrated (Table 2).

C_{18} neutral steroids were tentatively identified only in the sulphate-conjugated extracts obtained from umbilical venous plasma samples (Table 1). Using GC-MS analysis, an isomer of 5(10)-oestrenediol was tentatively identified, together with two structurally-related analogues which, from their fragmentation pattern, appeared to contain two and three sites of unsaturation, respectively (Fig. 2 a,b and c).

4. Discussion

The present studies have been concerned with the measurement of ten steroids in venous and arterial equine umbilical blood plasma samples. No steroids were identified as glucuronide conjugates in either arterial or venous plasma sample extracts, indicating that the foetus and placenta do not possess appreciable glucuronyltransferase activities. No marked differences in content of the various steroids were noted in plasma samples analysed between the four mares studied (Table 2). However, marked differences were demonstrated among the steroid contents of foetal arterial and venous plasma samples (Table 2). DHA and Δ^7-DHA were identified as the only C_{19} steroids in umbilical arterial plasma samples and were detected in unconjugated steroid extracts obtained from three of the four mares studied (Table 2). The other common C_{19} steroid, testosterone, was not identified in the current analyses. These findings are consistent with those of others [4–7]. However, although cells of the foetal gonad are capable of de novo synthesis of DHA from acetate [5], lack of 5-ene-3β-hydroxysteroid dehydrogenase/4,5-steroid isomerase means that the metabolism of DHA is minimised or non-existent [19,20]. It seems unlikely, therefore, that metabolites such as 4-androstenedione and testosterone, would be expected from this source.

The C_{21} compounds, pregnenolone, pregnanolone, pregnenediol and pregnanediol were also detected. Together with DHA and Δ^7-DHA, these four steroids dominated the chromatograms of the unconjugated residues obtained from umbilical arterial plasma samples. This confirms previous results [21] and suggests that either the foetus has low sulphotransferase activity or that the placenta has low sulphatase capacity. Moreover, the isomer of pregnanolone isolated by Holton et al. [22] had the 3β, 5α- configuration while it was the 5α- isomer of dihydroprogesterone that was detected by Hamon et al. [23], both these results being consistent with our present conclusions.

While some, or all, of these C_{19} and C_{21} steroids could be identified in the unconjugated extracts from umbilical venous plasma samples, their presence was appreciably less in the corresponding sulphate fractions, only traces being identified in the arterial plasma samples and none in the venous umbilical plasma samples (Tables 1 and 2). This finding lends weight to the suggestion that these compounds are utilised and not merely sulphated at the placental surface. It is of interest that, while preganedione was not identified in umbilical arterial extracts, low levels were detected in the unconjugated extracts from umbilical venous plasma samples, suggesting that this compound may arise from the placental surface. This has been proposed previously, further support being obtained from in vitro studies from which it was shown that pregnanedione is primarily biosynthesised by maternal endometrial tissues [22, 23].

Distinct differences in GC RT and mass spectral fragmentation patterns for the 17α- and β-reduced oestrogens were demonstrated, e.g. oestradiol, dihydroequilin and dihydroequilenin (Table 1). On the basis of this evidence oestradiol-17α, dihydroequilin-17α and dihydroequilenin-17α were all identified in extracts from both umbilical arterial and venous plasma samples. These data imply that the 17α-hydroxylated oestrogens represent the true oestrogens biosynthesised at the foetal placental surface. Further distinct differences in levels of the sulphate-conjugated oestrogens in the present study could be demonstrated between extracts from arterial and venous plasma samples in two of the four mares studied (Table 2). Whilst low levels of unconjugated and sulphate-conjugated oestrogens were detected in extracts from umbilical arterial plasma samples, all oestrogens returning from the placental surface were sulphate-conjugated. At 280 days gestation, 15, 100 and 16 fold-increases in the amounts of oestradiol-17α, dihydroequilin-17α and dihydroequilenin-17α, respectively (relative to the internal marker), could be estimated in the sulphate extracts from umbilical venous, compared with arterial, plasma samples (Table 2). These semi-quantitative data provide further evidence for the intrinsically high aromatase activity observed in equine placenta, e.g. [1,3]. However, our results disagree with those of previous authors, since we could only detect a small amount of oestrone in a few umbilical arterial plasma samples from one of the animals studied but no equilin, equilenin or 17β-reduced oestrogens. These data suggest that 17-oxidoreductase activity responsible for conversion of the 17α-oestrogens biosynthesised at the foetal placental surface, may occur extra-placentally.

There is clearly a major discrepancy between the in situ studies presented here and in vitro experiments in that 17α-hydroxylated oestrogens are found in the former methodology (Table 2) as opposed to those with 17β configuration in the latter [3]. Further oestradiol-17α, dihydroequilin-17α and dihyroequilenin-17α were found preferentially as sulpho-congugates in our in situ cannulation studies. As such, the negligible conjugation detected in the in vitro studies may account for the preferential 17β-reductive metabolism displayed. Assuming conjugation to be directed at C-17, then the lack of the sulphotransferase system in vitro may favour formation of the β configuration. In combination, these data suggest that placental sulphotransferase activity in vivo is directed preferentially at C-17 and requires an α stereochemistry. Using stable isotopic distribution patterns, we have shown earlier [3] that production of oestradiol-17β from incubation of testosterone in vitro using equine placental tissues pro-

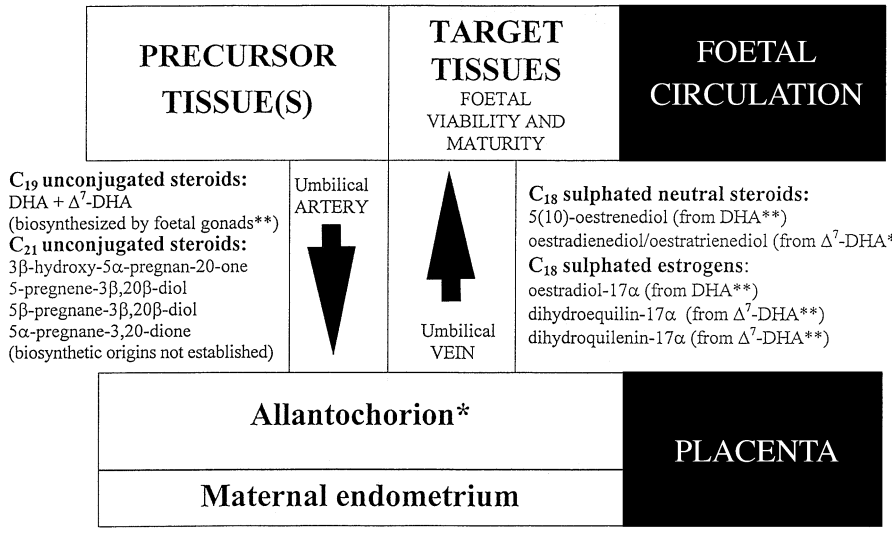

Fig. 3. Summary of steroid biosynthetic pathways in equine placental-foetal tissues. *Allantochorion, shown previously [3] to display high intrinsic aromatase ($P450_{arom}$) activity compared to that of maternal endometrium. ** Established in present work and/or previous authors.

ceeds via the formation of the intermediate ketone at C-17. As both DHA and Δ^7-DHA possess a ketonic function at C-17, it is reasonable to conclude that the aromatase system in equine placenta also displays a preference for a ketonic function at C-17. The lack of 17β-oestrogens in the umbilical arterial plasma samples suggests that the foetus is unable to contribute significantly to any 17-oxidoreductase activity.

The C_{18} neutral steroids were identified here as isomeric forms of 5(10)-oestrenediol, together with a compound recently characterised in our laboratory as 5(10),7-oestradienediol [Marshall, Mortishire-Smith, Gower and Houghton, unpublished] and its oxidative artefact, 5(10),7,9-oestratrienediol (so-called Diol of Heard's ketone) [23]. The presence of these three C_{18} neutral compounds, as sulphate conjugates, in umbilical venous, but not arterial, plasma extracts suggests that these compounds are biosynthesised, transferred and/or accumulated at the placental surface [Marshall, Mortishire-Smith, Gower and Houghton, unpublished]. Previously, 5(10)-oestrenediol has been identified as a product of in vitro demethylation without concomitant aromatisation of DHA, via a retro-aldol condensation mechanism [8]. We suggest, therefore, that the 5(10)-oestrenediol identified in the present studies arises from DHA identified in the umbilical arterial plasma. As 3-hydroxy-5(10),7,9-oestratrien-17-one has been isolated from in vitro incubation studies of equine placental tissues with Δ^7-DHA [9], it is conceivable that reduced 3-hydroxy-5(10),7,9-oestratrien-17-one i.e. 5(10),7-oestradienediol and consequently 5(10),7,9-oestratrienediol [24] are products of demethylation of Δ^7-DHA, which has been identified in the present studies as the other major C_{19} steroid in umbilical arterial plasmas. Cumulatively, these cannulation studies support the proposition that the placenta is able to display C_{19} demethylase without concomitant aromatase activity.

Significantly, the other ring A stabilised C_{18} neutral compounds, 19-nortestosterone (nandrolone) (M^+, m/z 375 [base peak m/z 254(17α)]) or 4-oestrene-3,17-dione (M^+, m/z 330, base peak m/z 299) were not identified in the current study of cord plasma samples. These data suggest that these steroids may be products of extra-placental metabolism. Again these data conflict with other research in which these compounds have been detected both in steroid profiling studies and as metabolites from incubation studies using testosterone and DHA with equine testes and placenta [8,10–13, 25]. As discussed above, the reason for this discrepancy may occur since we have used in situ cannulation techniques in our work.

In summary, the previously known gonadal-placental connection for DHA has now been shown to include also Δ^7-DHA. Further the present studies indicate the existence of a unique placental circulation for C_{19} and C_{21} steroids and C_{18} neutral and aromatic compounds. The overall pattern of equine placental-foetal steroid metabolism is summarised in Fig 3. High placental activities of aromatase, 5α-reductase, 3- and 20-oxidoreductase and oestrogen- and C_{18}- sulphotransferases are clearly necessary for these transformations to occur.

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