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GAS CHROMATOGRAPHIC DETERMINATION AND MASS SPECTROMETRIC IDENTIFICATION OF OESTROGENS IN NORMAL HUMAN PLACENTAL TISSUE AT TERM

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SUMMARY

Qualitative and quantitative studies of oestrogens by gas chromatography and mass spectrometry were carried out in extracts of eight human term placentae. Attempts were made to remove foetal blood from the placentae before analysis but some was still remaining. The identity of the following unconjugated oestrogens was established: oestrone, oestradiol, oestriol, 16α-hydroxyoestrone and 16-oxo-oestradiol. In addition, unconjugated 2-methoxyoestrone, 16-epioestriol, 17-epioestriol and 15α-hydroxyoestrone were detected. Despite the fact that many of these oestrogens were either identified or detected after hydrolysis of their conjugates, the results obtained suggest that only oestrone and oestradiol are present in significant amounts (more than 1 μg/kg) in conjugated form in placental tissue and that the other conjugated oestrogens found, derive from the residual foetal blood, in which their concentration is comparatively high.

INTRODUCTION

AT PRESENT it is generally accepted that the foetus and the placenta form two specialized compartments of an integrated functional unit [1–4] responsible for the production of oestrogens in pregnancy. The extensive literature on this subject has recently been reviewed by Diczfalusy and Mancuso [5]. One of the important functions of the placenta is to convert the neutral steroid precursors to oestrogens, which are secreted into both the maternal and foetal circulations. The main oestrogen formed in this way is oestriol. Studies dealing with the content of one or all three of the "classical" oestrogens, oestrone, oestradiol and oestriol, in placental tissue have been carried out by Collip [6], Butenandt and Browne [7], Westerfeld *et al.* [8], Huffman *et al.* [9], Diczfalusy [10], Diczfalusy and Lindqvist [11], Mitchell and Davis [12], Mitchell [13], and Schmidt-Elmendorff [14]. These oestrogens were found predominantly in unconjugated form. Subsequently, Diczfalusy and Halla [15] found an epioestriol-like Kober chromogen, which was provisionally identified as 16-epioestriol, in placental tissue. Unconjugated 16-oxo-oestradiol-17β was also detected in full-term placentae by Diczfalusy and von Münstermann [16].

The oestrogens in placental tissue have never been investigated with the modern techniques already used for the identification and quantification of these steroids in maternal and cord plasma, amniotic fluid and meconium [17–21]. The aim of the present investigation was therefore to obtain a more complete picture of the pattern of oestrogens in the foeto-placental unit.

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MATERIAL AND METHODS

Eight fresh normal placentae (for some data of the placentae, see Table 1) were prepared separately immediately after delivery. The maternal surface was scarified and the organ was rapidly perfused through the umbilical vein with 0·5 l of ice-cold 0·9% saline solution followed by 100–200 ml of air with a syringe under pressure. The placenta was dried between sheets of filter paper, suspended for a few min and then cut into pieces. Only chorionic placental tissue was

Table 1. Some data of the placentae investigated

	Weight of placenta		Foetal blood removed from placenta
	Fresh	After perfusion	
1	510 g	390 g	120 g
2	480 g	370 g	110 g
3	480 g	390 g	90 g
4	530 g	400 g	130 g
5	500 g	380 g	120 g
6	500 g	410 g	90 g
7	510 g	385 g	125 g
8	490 g	400 g	90 g

taken for analysis. The pieces were again dried with filter paper and 200 g of each placenta was homogenized with quartz sand and ice-cold distilled water and placed in a deep-freeze overnight for breakdown of the cell structure. Methanol was added to the homogenate in the ratio of 7:3 and the mixture was kept in a deep-freeze for 24–48 h. After centrifugation at − 18°C for 45 min the supernatant was decanted and the precipitate washed several times with cold 70% methanol, with effective stirring. The pooled methanolic extracts were evaporated to a small volume after addition of 50 ml of n-butanol to avoid foaming, and the precipitation of fatty material in cold methanol was repeated, but this time the precipitate was not washed. The final methanolic extract was then evaporated to dryness after addition of 50 ml of n-butanol, and the dry residue dissolved in 0·1 M phosphate buffer, pH 6·5, saturated with uric acid[22]. Unconjugated oestrogens were extracted with ether (3 × 1/1 vol), and the buffered extract containing the conjugates was then submitted to gel filtration as described by Beling[23]. The further purification and separation of the oestrogens were done according to Adlercreutz and Luukkainen[24, 25]. All details, including some essential practical hints and some modifications of the original procedure, have been described by Adlercreutz [26].

In previous investigations[17–21] the procedure used has been found to be highly specific for the compounds investigated and their behaviour in the various steps contributed additional evidence of their identity. The identity of the oestrogens was finally established in repeated analyses by combined gas chromatography–mass spectrometry (g.c.–m.s.). Thus after hydrolysis, the ketonic oestrogens are separated from the nonketonic ones by the Girard separation, carried out essentially as described by Givner *et al.*[27]. The 2-methoxyoestrone-oestrone fraction is taken through a solvent partition, and chromatography on partially deactivated alumina, and then after formation of the trimethylsilyl ether (TMS) derivatives (hereafter called silylation) of the two oestrogens the mixture is

separated by gas chromatography (g.c.). The fraction containing the ring D-ketolic oestrogens (16α-hydroxyoestrone, 16β-hydroxyoestrone, 16-oxo-oestradiol-17β, 15α-hydroxyoestrone) is carried through a solvent partition, two paper chromatographic systems, and a chromatography on partially deactivated alumina, and is then submitted to g.c. separation on a QF-1 liquid phase after silylation of the 4 oestrogens. The oestradiol fraction, usually containing a great number of different oestradiols, is carried through a solvent partition, methylated and submitted to chromatography on partially deactivated alumina[28]. The various oestradiols are then separated by g.c. on a SE-30 liquid phase after silylation of the monomethylated oestradiols. The oestriol fraction is carried through two solvent partitions and then chromatographed on partially deactivated alumina, methylated and again chromatographed on alumina. Finally it is submitted to g.c. in the form of the bistrimethylsilyl ether derivative of the monomethylated compound. The two isomeric oestriols, 16-epioestriol and 17-epioestriol, after conversion to their acetonide derivatives, are separated from oestriol by extraction with chloroform. The two 16,17-acetonides are then chromatographed on partially deactivated alumina and, after silylation, by g.c. in the form of the monotrimethylsilyl ether derivatives of the acetonides.

Formation of derivatives

Methylation was done according to Brown[28], acetonide formation according to McClosky and Clelland[29], and silylation according to Luukkainen *et al.*[30], but in dry pyridine instead of chloroform.

Paper chromatography

This was carried out for the ring D-ketolic oestrogens on formamide-impregnated paper. As the first system, cyclohexane-toluene (1 : 1 v/v) formamide is used overnight (16 h). The paper was dried and transferred to another paper chromatography tank and run for 6 h at the same temperature in a chloroform/formamide system[31].

Alumina chromatography

Acid alumina (Merck AG, Darmstadt, Germany) washed with ethyl acetate and reactivated was used. The preparation and standardization of the alumina columns and the solvent systems used for the various oestrogen fractions have been described elsewhere[26, 32].

Measurement of radioactivity

This was measured in a Wallac DECEM-NTL 314 automatic liquid scintillation counter (Wallac Oy, Turku, Finland). Bray's liquid scintillator[33] was used. No significant quenching was observed in the highly purified samples measured.

Gas–liquid chromatography

Two Hewlett–Packard model 400 and 402 gas chromatographs with hydrogen flame ionization detectors were used. The glass tubes were U-shaped with a length of 2–4 m and i.d. of 2·5 mm. As stationary phases 2·5% QF-1 on 80/100 mesh Gas-Chrom Q (Applied Science Laboratories Inc., State College) and 1% SE-30 on 80/100 mesh Gas-Chrom Q were used. The first mentioned coated support for column packing was prepared as described by Haahti[34]; the SE-30 column packing is commercially available. The tubes were packed as described by

Vanden Heuvel and Horning[35] modified by applying suction at the detector end of the tube. The hydrogen flame was supported by air or oxygen and nitrogen was used as carrier gas. The samples were injected into the columns with Hamilton micro-syringes.

Gas chromatography–mass spectrometry

The mass spectra were recorded with an LKB Gas chromatograph–mass spectrometer combination instrument (LKB-Produkter AB, Bromma, Sweden). As stationary phases 2–3% SE-30 and 1–2% QF-1 on 100/120 mesh Gas Chrom Q were used. The QF-1 column was maintained at about 210°C and the SE-30 column at 240–250°C. The temperature of the separator and the flash-heater was kept at 10 and 30°C above the column temperatures, respectively. The ionizing energy was 70 eV in all experiments. Multiple mass spectra were recorded in all instances when there was doubt about the homogeneity of the g.c. peak corresponding to the compound investigated. One mass spectrum was always recorded at exactly the same retention time as the corresponding reference standard. The mass spectra of all oestrogens studied have been published and discussed previously[17, 20, 21, 24, 36].

Criteria used for establishing the identity of the unknown steroids with the reference standards

Owing to the very small amounts of oestrogens present in human placental tissue at term, isolation of the compounds in crystalline form was not possible. However, the steroids were separated from each other and "isolated" by g.c. and analysed with the mass spectrometer as each compound emerged from the g.c. column. As described above, various characteristic derivatives of the compounds were first formed during the purification procedure and finally the compounds were silylated immediately before g.c. Identity of the unknown steroid with the corresponding reference standard was regarded as established if (1) the unknown steroids behaved identically with the reference standard during the various chromatographic and other steps of the isolation procedure described above (for details see [26]) and (2) if the final derivative of the unknown compound had an identical retention time with the corresponding derivative of the reference compound on the g.c. column(s) found by experience to give the most specific result. If necessary, the retention time was checked on both a non polar and a polar liquid phase. (3) Finally, for identity, the mass spectrum of the unknown compound had to show the molecular ion and at least 5 of the most characteristic fragments with correct intensities and no significant additional peaks in the higher mass region. A compound was said to have been detected if criteria 1 and 2 were satisfied but the mass spectrum showed some additional peaks due to contamination with other compounds, usually a small amount of some neutral steroid. Mass spectrometric analyses were carried out for every oestrogen fraction of all eight placentae investigated. Because the identification technique was previously used for the investigation of oestrogens in many biological fluids, the reader is referred to these for further information.

Quantification

Small and irregular gas chromatographic peaks were not quantified. The method has previously been used for the determination of oestrogens in bile,

amniotic fluid, cord plasma and maternal plasma and urine [19, 20, 24]. The processing of the samples was found to be especially difficult owing to the large amounts of lipids in the placental tissue. Especially the fraction containing the unconjugated steroids presented great problems. Therefore radioactive internal oestrogen standards were added both to the ether extract containing the unconjugated steroids and to the extract containing the conjugated oestrogens after gel filtration, and recovery was calculated by liquid scintillation counting immediately before g.c. The following radioactive standards were used: [6,7-^{3}H]-oestrone, S.A. 50 mCi/mmol; [6,7-^{3}H]-oestradiol-17β, S.A. 47 mCi/mmol; [6,7-^{3}H]-oestriol, S.A. 37 mCi/mmol (all from N.E.N. Corp. Boston, U.S.A.). The recovery for oestrone ranged from 21 to 66%, for oestradiol-17β from 23 to 62% and for oestriol from 38 to 89%. Radioactive standards were not available for the other oestrogens measured. Therefore the recoveries were determined by adding non-radioactive reference compounds to human male plasma samples, processing these in the same way and simultaneously with the placental extracts, and correcting the values according to the results obtained. The recoveries were 62 and 70% for 16α-hydroxyoestrone, 73 and 79% for 16-oxo-oestradiol-17β, 62 and 70% for 15α-hydroxyoestrone, 47 and 50% for 16-epioestriol and 35 and 35% for 17-epioestriol. All quantitative analyses were carried out by an inexperienced person and because of this and the special problems involved in the analysis of placental tissue oestrogens the results for the "non classical" oestrogens should be regarded as semiquantitative.

Haemoglobin in cord blood and placental homogenate was determined with the cyanmethaemoglobin method [37].

RESULTS

By the criteria described above, the identity of the following unconjugated oestrogens was established in at least one placental extract (all results are given in Table 2): oestrone, oestradiol-17β, oestriol, 16α-hydroxyoestrone and 16-oxo-oestradiol-17β. In addition, 2-methoxyoestrone, 16-epioestriol, 17-epioestriol and 15α-hydroxyoestrone were detected in the unconjugated oestrogen fraction in at least one placental extract.

The identity of the following oestrogens was established in the fraction containing conjugated oestrogens in at least one placental extract: oestrone, oestradiol-17β, oestriol, 16α-hydroxyoestrone, 16β-hydroxyoestrone, 16-oxo-oestradiol-17β and 15α-hydroxyoestrone. In addition, conjugated 2-methoxyoestrone and 16-epioestriol were detected in at least one placental extract. One placental extract contained a compound giving the fragmentation pattern of the mono TMS derivative of 17-epioestriol acetonide, but the mass spectrum contained several additional peaks in the higher mass region. If present in the fraction containing conjugated oestrogens, 17-epioestriol must have been present in very small amounts.

The quantitative results obtained for the individual placentae are presented in Table 3. Because every fraction was analysed by mass spectrometry, it was possible to exclude all values that were found to contain more compounds than the one investigated. In Table 3, those values which were found by mass spectrometry to be too high owing to contamination of the peak with some other compound were eliminated and marked "n.d." (not determined). If the g.c. peaks were too small for quantification the same letters were used. Because of the mass

Table 2. Oestrogens identified (= Ident.) or detected (= Detect.) in the 8 placental extracts investigated by gas chromatography–mass spectrometry. Number of placentae in which the estrogens were either identified or detected are indicated in brackets

	Oestriol	Oestrone	2-Methoxy-oestrone	Oestradiol-17β	16-epi-oestriol	17-epi-oestriol	16α-hydroxy-oestrone	16β-hydroxy-oestrone	16oxo-oestradiol-17β	15α-hydroxy-oestrone
Unconjugated fraction	Ident. (8)	Ident. (7) Detect. (1)	Detect. (1)	Ident. (1) Detect. (5)	Detect. (3)	Detect. (1)	Ident. (5) Detect. (3)		Ident. (5) Detect. (3)	Detect. (2)
Conjugated fraction	Ident. (8)	Ident. (5) Detect. (3)	Detect. (2)	Ident. (3) Detect. (3)	Detect. (1)		Ident. (2) Detect. (6)	Ident. (1) Detect. (2)	Ident. (6) Detect. (2)	Ident. (2) Detect. (5)

Table 3. Gas chromatographic determination of unconjugated and conjugated oestrogens in human term placental tissue. The values were corrected for experimental losses during the procedure and expressed as μg/kg placental tissue

Placenta specimen	Oestriol		Oestrone		Oestradiol-17β		16α-hydroxyoestrone		16-oxo-oestradiol-17β	
	Unconjug.	Conjug.	Unconjug.	Conjug.	Unconjug.	Conjug.	Unconjug.	Conjug.	Unconjug.	Conjug.
I	34	16	4·1	11	35	2·5	n.d.*	8·4	n.d.	n.d.
II	30	16	4·5	6·5	11	2·2	4·1	1·5	2·4	n.d.
III	n.d.	25	12	8·6	11	3·5	n.d.	5·7	n.d.	n.d.
IV	n.d.	17	8·7	13	16	n.d.	20	4·2	n.d.	n.d.
V	40	10	5·8	8·1	n.d.	6·3	8·3	6·7	5·7	n.d.
VI	67	41	33	6·6	8	13	27	12	32	n.d.
VII	86	21	n.d.	9·6	n.d.	2·5	14	3·3	23	n.d.
VIII	55	58	n.d.	12	n.d.	n.d.	7·5	9·7	13	n.d.

*n.d. = not determined.

spectrometric analysis of every fraction, the values can be regarded as representing a highly-specific determination of the compound but, as pointed out, methodological difficulties and lack of radioactive standards for the non-classical oestrogens led to semiquantitative results for these compounds.

DISCUSSION

In all previous investigations on placental oestrogens, with the exception of that of Schmidt-Elmendorff[14], the whole placentae have been extracted, which means that a considerable proportion of the oestrogens found was derived not from placental tissue but from foetal blood. Schmidt-Elmendorff perfused the placentae with liquid paraffin, but he did not scarify the tissue to enable the blood to flow better.

Although in the present study specific determination of Hb in placental homogenate was not possible due to some non specific absorption the results indicated that significant amounts of foetal blood still contaminated the placental tissue. The results obtained should therefore be judged in the light of this observation.

The results of previous assays of oestrone, oestradiol and oestriol and the present values are depicted in Table 4. It is difficult to compare the values with each other, both because the oestrogens in foetal blood have been co-estimated to varying degrees and because of differences in methodology. The results obtained in the present investigation are much lower than the previous ones, with the exception of the values for conjugated oestrogens, which are similar to those obtained by Diczfalusy[10] and Mitchell and Davis[12]. The proportions of the various unconjugated oestrogens are similar to those found by Huffman *et al.*[9] but the values obtained by these latter workers by bioassay were 3–4 times higher. However, when allowance is made for the probably comparatively high content of foetal blood in the placentae investigated by Huffman *et al.*[9], their values are seen to fall within the range of values found in the present investigation.

As previously noted by other investigators, the concentration of conjugated oestrogens in placental homogenate is low. Our results indicate that conjugated oestrone and oestradiol are present in significant amounts but that conjugated oestriol is absent. The presence of the latter form of oestriol seems to be entirely due to the presence of remaining foetal blood in the homogenate, because the concentration of conjugated oestriol in foetal blood is very high (Table 5).

Endogenous oestrogens other than the three "classical ones" have never previously been determined in placental extracts. In the present study reliable estimates could be obtained for 16α-hydroxyoestrone and 16-oxo-oestradiol, because they were the only "non-classical" oestrogens present in measurable amounts with the g.c. technique used. The concentrations found for these two oestrogens indicate that they are present in human placental tissue at term. However, the presence of the conjugated forms of these two ketolic oestrogens may be completely explained by the presence of foetal blood in the homogenate analysed, because their concentration in cord blood is considerable (Table 5). The other unconjugated oestrogens identified with certainty by g.c.–m.s., but not present in sufficient amounts for quantitative measurements by g.c., must really be present in placental tissue, because their concentration in cord plasma is very low. For the other conjugated oestrogens identified or detected (not mentioned above) it is reasonable to assume that they are derived from foetal blood. Thus we may conclude that only oestrone and oestradiol occur in substantial amounts (more than

Table 4. Values found by different authors for oestrone, oestradiol-17β and oestriol in placental tissue (μg/kg)

Authors	Unconjugated			Conjugated			Remarks
	Oestrone	Oestradiol-17β	Oestriol	Oestrone	Oestradiol-17β	Oestriol	
Huffman *et al.*[9]	35	38	140				Values uncorrected for procedural losses
Diczfalusy[10]	47	3·1	125	2·5	1·5	31·4	Values uncorrected for procedural losses
Mitchell and Davis[12]	86	25	190	21	8	44	Values uncorrected for procedural losses
Mitchell[13]	584	170	678				Values corrected for procedural losses
Diczfalusy and Lindqvist[11]	31	129	228				Values uncorrected for procedural losses
Heusghem[38]	92	15	223				
Schmidt-Elmendorff[14]	41	101	180				Values uncorrected for procedural losses
Present investigation	11	16	52	9	4	26	Values corrected for procedural losses

Table 5. Mean values and ranges for 5 oestrogens determined in placental tissue, cord and maternal plasma, and amniotic fluid at term

	Placenta (μg/kg)		Cord plasma* (μg/l)		Maternal plasma* (μg/l)		Amniotic fluid* (μg/l)	
	Unconjug.	Conjug.	Unconjug.	Conjug.	Unconjug.	Conjug.	Unconjug.	Conjug.
Oestriol	52 (30–86)	26 (10–58)	140 (95–206)	1030 (347–1910)	6 (3–9)	124 (105–142)	56 (19–146)	931 (197–1600)
Oestrone	11 (4–33)	9 (7–13)	26 (17–38)	28 (14–38)	10 (7–14)	80 (70–85)	6 (1–11)	8 (2–12)
Oestradiol-17β	16 (8–35)	4 (< 1–13)	7 (1–19)	3 (< 1–5)	15 (7–22)	5 (3–6)	+ (< 1–3)	1 (< 1–3)
16α-hydroxyoestrone	10 (< 1–27)	6 (2–10)	4 (2–6)	46 (20–66)	2 (< 1–6)	40 (20–58)	2 (< 1–4)	25 (14–53)
16-oxo-oestradiol	15 (2–32)	+	6 (2–14)	79 (22–201)	2 (1–5)	20 (11–28)	3 (< 1–9)	27 (13–46)

*Data from Adlercreutz and Luukkainen [20].

1 μg/kg) in conjugated form in human placental tissue at term. The data in Table 5 show that the concentrations of all 5 unconjugated oestrogens quantified in placental tissue are equal to or higher than the levels in maternal plasma. However, only the concentrations of oestradiol, 16α-hydroxyoestrone and 16-oxo-oestradiol are higher in placental tissue than in cord plasma. The concentrations of unconjugated oestrone and oestriol are 2·5–3 times higher in cord plasma as in placental tissue, which means that if they are secreted into the foetal circulation this occurs against a concentration gradient. The similar concentrations of unconjugated oestrone and oestradiol in placental tissue and maternal plasma suggest that the placenta maintains the level of these oestrogens in maternal plasma. In view of this observation and the fact that a considerable part of these oestrogens is derived from precursors produced by the maternal adrenals (for a discussion, see [19]), it seems likely that unconjugated oestrone and oestradiol in maternal plasma mainly reflect placental function, and that their assay is not suitable for foetal monitoring.

The sum of the concentrations of unconjugated oestrone and oestradiol in placental tissue and cord plasma is approximately the same, suggesting that oestradiol, which is obviously secreted to a lesser degree into the foetal than into the maternal circulation[39], is rapidly converted to oestrone. The much higher concentration of unconjugated oestriol in cord plasma as compared to placental tissue is somewhat surprising and suggests that at term much of the oestriol is primarily formed in the foetal organism. This may occur from oestrone and oestradiol[40, 41] and probably also from 16α-hydroxyoestrone produced from 16α-hydroxyandrostenedione in the placenta. Aromatization of neutral steroid precursors in the foetal liver is also one possible source of oestriol[41]. Oestriol has not been measured in foetal liver and adrenals at term. It is therefore impossible to speculate about the proportions in which the placenta and the foetal organism contribute to the production of oestriol with regard to the final enzymatic step at term. It should be mentioned in this connection that Maner *et al.*[43] could not find evidence for placental oestriol secretion into the umbilical vein. However, our data seem to indicate that the final step in the biosynthesis of oestradiol, 16α-hydroxyoestrone and 16-oxo-oestradiol mainly takes place in human placental tissue at term. According to our present view, this step is the aromatization of the corresponding neutral steroid precursors which occur in high concentrations in cord plasma[44, 45].

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