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Isolation and characterisation of a C_{18} neutral steroid, oestra-5(10),7-diene-3,17-diol, from pregnant mare urine and allantoic fluid. Facile oxidation to yield oestra-5(10),6,8-triene-3,17-diol (diol of Heard's ketone)

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Abstract

Oestradiene-3,17-diol and oestratriene-3,17-diol (or the diol of Heard's ketone (3-hydroxy-5(10),6,8-oestratriene-17-one) have been extracted on a large scale from pooled urines and allantoic fluid obtained from pregnant mares. Initial purification was achieved using column chromatography, and further purification by high performance liquid chromatography or silver nitrate (argentation) thin layer chromatography. The steroids were characterised using gas chromatography–mass spectrometry. Positions of the double bonds in ring B of oestradienediol were deduced on the basis of results of ultraviolet (UV) and nuclear magnetic resonance (NMR) spectroscopy, hydrogenation, and incubation studies with the enzyme 5-ene-3β-hydroxysteroid dehydrogenase/steroid-4,5-isomerase. The reference steroid, 5,7-cholestadien-3β-ol (7-dehydrocholesterol), with its conjugated double bond system, behaved entirely differently to oestradienediol, consistent with the latter having no conjugated system. These data, together with detailed results of NMR studies, have led us to designate the positions of the double bonds in oestradienediol as 5(10),7-. The instability of the dienediol became apparent when the steroid was converted to its bis-trimethylsilyl (TMS) ether. The phenomenon was exacerbated when derivatisation was performed at elevated temperatures or when the fraction containing the dienediol was stored at 4°C prior to being derivatised. The facile oxidation product was shown to be 5(10),6,8-oestratriene-3,17-diol, implying that the two steroids are related and, furthermore, that all the sites of unsaturation are in the B ring. Because of the facile oxidation of oestradienediol to oestratrienediol (the diol of Heard's ketone), we propose, that this, and by implication, Heard's ketone itself, are artefacts of the isolation procedures which were utilised in the original studies. A possible mechanism is proposed for the biosynthesis of 5,7-oestradienediol from a ring-B unsaturated C_{19} compound, involving C_{19} demethylation without aromatisation.

Keywords: GC–MS; NMR; Oestra-5(10),7-diene-3,17-diol; Oestra-5(10),6,8-triene-3,17-diol; Heard's ketone

Abbreviations: Androstenediol, androst-5-ene-3β,17β-diol; Dehydroepiandrosterone, DHA, 3β-hydroxyandrost-5-en-17-one; 7-Dehydrocholesterol, 3β-hydroxycholesta-5,7-diene; Diol of Heard's ketone, oestratrienediol, oestra-5(10),6,8-triene-3,17-diol; 3β-HSD-isomerase, 5-ene-3β-hydroxysteroid dehydrogenase-4,5-isomerase (EC 1.1.3.6); GC–MS, gas chromatography–mass spectrometry; Heard's ketone, 3-hydroxyoestra-5(10),6,8-trien-17-one; HPLC, high pressure liquid chromatography; I.S., internal standard; Oestrenediol, oestr-5(10)-ene-3,17-diol; Oestradienediol, oestra-5,7-diene-3,17-diol; TIC, total ion chromatogram; TEAP-LH20, triethylaminohydroxypropyl-LH20; TLC, thin layer chromatography; UV, ultraviolet.

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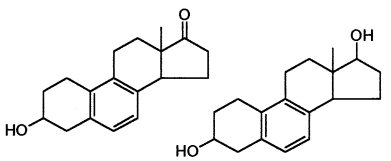
1. Introduction

The urine of pregnant mares contains significant amounts of the 'classical' oestrogens, oestradiol-17β and oestrone, together with ring-B unsaturated oestrogens, equilin and equilenin and their 17α- and 17β-reduced counterparts [1]. These urines are thus valuable sources of these hormones for use in clinical replacement therapies. These do not occur in stallion urine nor in normal human pregnancy urine. The circulating concentrations of the two groups of oestrogens, 'classical' and ring B -unsaturated, appear to rise and fall independently of each other during equine pregnancy [1]. The maintenance of such striking non–synchronous circulating concentrations suggested that the two groups of oestrogens might arise from different biosynthetic precursors. One such, suggested many years ago [2], is 3β-hydroxy-5,7-androstadien-17-one, which could conceivably be derived by side-chain cleavage, from 7-dehydrocholesterol (also a 5,7-diene structure). It was suggested that the 3β-hydroxy-5,7-androstadien-17-one could then be aromatised by the equine placenta to yield equilin (3β-hydroxy-1,3,5(10),7-oestratetraen-17-one [2].

In our studies presented here, two other related ring-B unsaturated steroids, 5(10),7-oestradienediol and oestratrienediol (see structure below) have been identified not only in the urine of pregnant mares but also as major constituents of allantochorionic fluid extracts. Oestratrienediol is, in fact, the so-called diol of Heard's ketone. The allantoic cavity is unique to the physiology of equine pregnancy inasmuch as it acts as a sink for waste products from the foetal kidneys [3]. Thus, contamination of the amnion in which the foetus is cushioned throughout gestation, is kept to a minimum. In a recent publication [4] we have identified (tentatively) mono, di and oestratriene-3,17-diols (ring B saturated) C_{18} neutral steroids in umbilical venous plasmas from umbilical in situ cannulation studies. As several isomers of the oestrenediols are theoretically possible, we have now used various chemical, spectroscopic and biochemical methods to designate the likely position of the double bonds in ring B of oestradienediol.

In preliminary work [5,6], we have quantified the C_{18} diols, oestrenediol, oestradienediol and oestratrienediol in amniotic and allantochorionic fluids throughout several equine pregnancies. Our results suggest a positive correlation between quantities of these steroids (relative to those in normal pregnancies) and foetal viability. The biological significance of the C_{18} diols, including oestradienediol, is thus highlighted and has largely given rise to the present studies. Since our work was undertaken, isomeric oestra-5(10),7-dienediols have been synthesised by chemical methods and shown to have IC_{50} values of 2×10^{-7}M and 1.5×10^{-7} M for the 3α- and 3β- isomers, respectively in an oestrogen binding study [7].

In 1940, Heard and Hoffman [8] isolated from the urine of pregnant mares a C_{18} neutral steroid that contained sites of unsaturation in the B-ring. This was identified as 3-hydroxy-oestratrien-17-one and called Heard's ketone. Later, the reduced compound (the diol of Heard's ketone), 5(10),6,8-oestratriene-3,17-diol, was identified.



Heard's ketone Diol of Heard's ketone

The techniques used for extraction and purification included the use of strong acids and alkalis with prolonged exposure to extremes of temperature [8]. More recently, during steroid profiling studies and GC–MS analysis, the presence of Heard's ketone has been confirmed in extracts of urine from stallions and pregnant mares [4,9]. In addition, two other C_{18} steroids containing 1 and 2 sites of unsaturation (compared to 3 sites in Heard's ketone) and the diol of Heard's ketone have been identified [4,9]. During our present work, we have found incidentally that the oestradienediol, which was being isolated and characterised, was converted quite easily to oestratrienediol, and we have explored this phenomenon.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all materials were obtained from Sigma-Aldrich Chemical Co., Fancy Rd, Poole, Dorset, UK, while HPLC grade solvents were obtained from Rathburn Chemicals Ltd, Walkerburn, Scotland. Precoated plastic-backed TLC plates (Kieselgel 60 F_{254}) and Kieselgel H-60 powder were purchased from BDH Chemical Co., Poole, UK. C_{18} Sep-Pak cartridges were purchased from Waters Chromatography Division, Millipore Corporation, Milford, MA.

2.1.1. Steroids

[16,16,17-$^2H_3$]5α-Androstane-3α,17β-diol was prepared and used as an internal marker in GC–MS studies [4,10]. Any authentic reference steroids required were obtained from either Sigma-Aldrich Chemical Co., or Steraloids (UK) Ltd, New Barnet, Herts., UK.

2.1.2. Sources of biological fluids

Pregnant mare urine and allantoic fluids were collected from a series of seven pregnant thoroughbred mares and four ponies. The gestational ages at the time

of sample collection ranged from 100 to 300 days, ascertained by last known covering dates and ultrascan techniques.

2.2. Methods

2.2.1. Isolation of C_{18} neutral steroids

2.2.1.1. Pregnant mare urine. In previous studies using pregnant mare urine, a complex profile of C_{19} and C_{18} neutral and phenolic steroids was identified in free, glucuronide and sulphate-conjugated fractions [4]. The following protocol was utilised for large-scale extraction of urine and allantoic fluid:

The pooled urine (500 ml) was adjusted to pH 6.8 and hydrolysed (*Escherichia coli*, 100 Units, 50°C, 2 h). The hydrolysed urine was applied to a column of XAD-2 resin [11] (column diam. 35mm, bed height 12.5cm); the column was washed with deionised water (1000 ml) and the absorbed material was eluted using methanol (500 ml). The methanol was removed in vacuo and the residue was solvolysed (acidified ethyl acetate: methanol 8:2 v/v, 50 ml, 50°C, 2 h). The solvent was then neutralised using saturated $NaHCO_3$ solution prior to its removal in vacuo. The residue was reconstituted in methanol:chloroform:water (80:15:30 by vol, 500 μl) and neutral and phenolic steroids were separated by multiple chromatographic steps using TEAP-LH20 column chromatography [12].

The neutral fraction was subjected to normal phase (silica Kieselgel H60) column chromatography (25 g) using chloroform:ethanol (95:5, v/v, 250 ml), followed by ethyl acetate:hexane (4:6 v/v, 200 ml) as eluting solvents. Fractions (10 ml) were collected. Solvents were removed in vacuo and residues subjected to TLC analysis and/or GC–MS to determine the presence of oestradienediol [4]. After column chromatography, further purification of the urinary extract was achieved by HPLC using an ODS semi-preparative column (20 cm × 5 mm, i.d. Chrom-Sep) interfaced to a fast scanning UV detector (Chrom-a-scope, Barspec). An aliquot (100 μl) of the neutral sample, dissolved in methanol–water (1:1 v/v), 100 μl, was introduced via a Rheodyne loop injector port. Gradient elution was carried out at a flow rate of 2 ml/min from 50 to 100% methanol over 7 min, followed by an isocratic step of 13 min. Fractions (0.5 ml) were collected and evaporated to dryness. Portions were derivatised and analysed by GC–MS. A fraction (F22), was obtained from which C_{19} steroids had been successfully separated, and appeared to contain only oestradienediol and oestratrienediol (see Section 3, Fig. 1).

2.2.1.2. Allantoic fluid. Previous work involving steroid profiling had shown that only very small amounts of steroid glucuronides were present in allantoic fluids [4]. In view of this, allantoic fluids were pooled (1000 ml) and subjected to XAD-2 extraction without initial enzymic hydrolysis and elution performed with methanol (as above for urine). After solvolysis and neutralisation, a fraction was obtained by column chromatography which contained the mono-, di- and trienediol C_{18} compounds (see Section 3, Fig. 1).

An earlier method, called argentation TLC [13,14], was then used to separate effectively the mono- and di-unsaturated C_{18} steroids in these extracts. Plastic-backed TLC plates of silica impregnated with $AgNO_3$ were prepared [14] and argentation TLC of extracts and marker steroids was carried out using chloroform:ethyl acetate (1:1 v/v). Marker steroids were detected using an acidified ethanolic spray (ethanol:water:sulphuric acid (36:4:10 by volume) [15]. Recovery of the steroids from the non-sprayed portion of the TLC plate was achieved as described earlier [14]. The steroids were then extracted with diethyl ether. The organic phase was aspirated and dried over $NaSO_4$ prior to the removal of diethyl ether under oxygen-free nitrogen. Using these methods, Fractions 4 and 5 were obtained (see Section 3, Fig. 1).

2.2.2. Derivatisation and GC–MS analysis

O-Methoximes(MO)/bis-trimethylsilyl(TMS) ethers were prepared at room temperature overnight, because of the facile oxidation of oestradienediol which may have been present. Purification of derivatives was achieved by chromatography on a short column of Sephadex LH20 [4]. The residues were dried and then dissolved in undecane containing the bis-TMS ether derivative of [16,16,17-$^2H_3$]5α-androstane-3α,17β-diol at a concentration of 10 ng/μl which acted as an internal standard in the GC–MS studies. These were performed as before [4] except that the GC oven temperature was set initially at 150°C, then programmed to rise, 2 min after injection, by 20°C/min to 220°C then by 5°C/min to a final temperature of 300°C (held for 5 min).

2.2.3. Other analyses

2.2.3.1. Hydrogenation. Portions of fraction F22, purified from the urinary extract (see above), and of 7-dehydrocholesterol (for comparison), were dissolved in methanol (300 μl) containing rhodium on alumina (5 mg). Hydrogen gas was slowly sparged through this suspension at 0°C for varying periods of time. After reaction, the catalyst was sedimented by centrifugation (500 *g*, 5 min) and the methanolic solution containing hydrogenation products, was aspirated. These were derivatised for GC–MS analysis as above.

2.2.3.2. Isomerisation reaction. Portions of F22 and authentic reference steroids, 5(10)-oestrenediol and de-

hydroepiandrosterone (DHA) were subjected to isomerisation using 5-ene-3β-hydroxysteroid dehydrogenase (EC 1.1.3.6) ex *Brevibacterium stercolicum*. The reaction mixture containing the enzyme (1 mg), in NADPH regenerating system (5 ml of 100 mM phosphate buffer, pH 7.4, containing $NADP^+$, 1 mM, glucose-6-phosphate, 6 mM and glucose-6-phosphate dehydrogenase, 2 U) was incubated for 90 min at room temperature. Products were then extracted using activated C_{18} Sep-Pak cartridges, steroids being eluted with diethyl ether (10 ml). The solvent was dried (Na_2SO_4) and removed under oxygen-free nitrogen. The steroid products were derivatised and analysed by GC–MS as described above.

2.2.3.3. Nuclear Magnetic Resonance (NMR). NMR studies were performed on fractions 4 and 5, which had been obtained from allantoic fluid (as above). As silver ions interfere with NMR spectral interpretation, the residues from argentation TLC were cleaned up by C_{18}

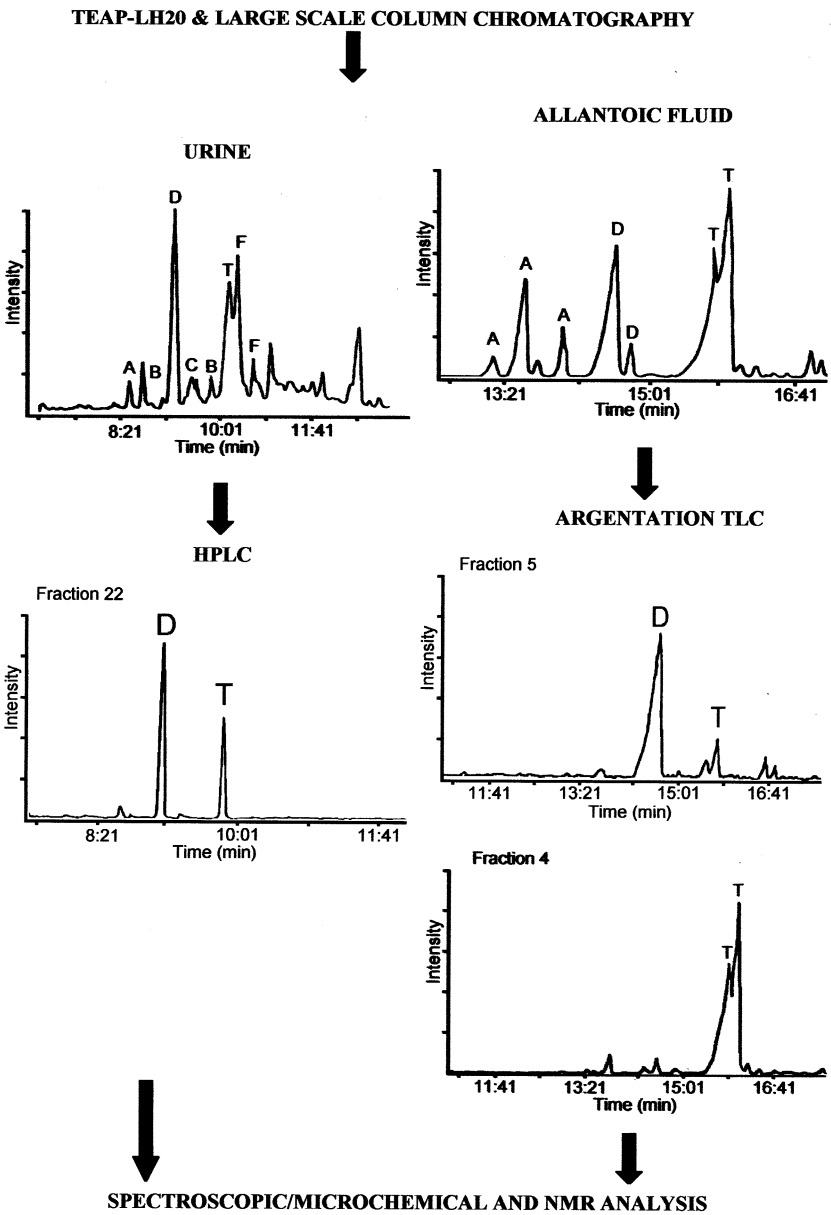


Fig. 1. Isolation and purification of oestradienediol and oestratrienediol from equine pregnancy urine and allantoic fluid. Initial chromatographic separation (see Section 2) of urine extracts resulted in a fraction, which on GC–MS analysis gave a total ion chromatogram (TIC) shown top left. Steroids identified were: A, oestrene-3,17-diol; B, androstane-3,16-diol; C, androstane-3,17-diol (2 isomers); D, oestradienediol; E, 5-androstene-3,17-diol (2 isomers) and T, oestratrienediol. HPLC resulted in various fractions, one of which (F22) was shown by GC–MS to contain only D and T. Initial chromatographic separation of allantoic fluid extract resulted in a fraction with a TIC as shown top right, and was found to contain steroids: A, oestrene-3,17-diol (3 isomers); D and T. Further purification by argentation TLC yielded fractions 4 and 5, which respectively contained D plus T and T only. Fractions 22, 4 and 5 were further subjected to a range of analytical methods.

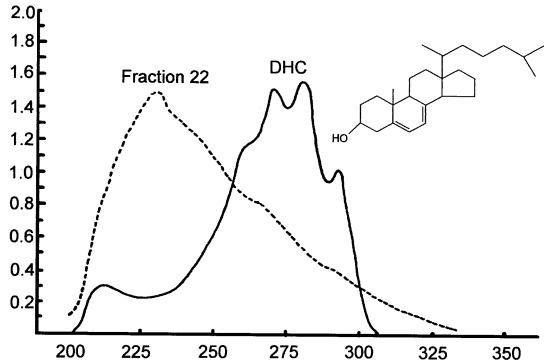


Fig. 2. UV spectroscopy of Fraction 22, obtained from equine pregnancy urine as described in Section 2 and Fig. 1. The UV spectrum for the 5,7-conjugated diene, 7-dehydrocholesterol, is shown for comparison; note, in this case, the 3 characteristic absorbance peaks at 272, 282 and 292 nm.

Sep-Pak extraction. The samples were dissolved in phosphate buffer (50 mM, pH 7.4, 5 ml) and applied to individual activated cartridges. After the cartridges had been dried with hexane (5 ml), the steroids were eluted with diethyl ether (10 ml). The ether was removed using oxygen-free nitrogen and the sample extracts dissolved in 99.999% deuterochloroform ($CDCl_3$) for NMR analysis.

Separate one-dimensional (1D) spectra were acquired on either a WM250 or AM400 spectrometer (Bruker) over the spectral width of 2500 or 4000 Hz with quadrature detection. Chemical shifts were measured relative to residual $CDCl_3$. The DQF-COSY spectrum of the extract containing the oestradienediol was acquired in the phase-sensitive mode using quadrature detection in f_2 and with TPPI for f_1. The data set resulting from 512 increments of t_1 was zero filled to 1024 points, with each FID composed of 2048 data points. The recycle delay between transients was 1.0 s with 64 transients being recorded for each increment of t_1. The final data set was subjected to shifted sine-bell apodisation before Fourier transformation.

The extract containing the oestradienediol was re-analysed 10 days and 4 weeks later.

3. Results

Analysis by GC–MS of urinary extracts from pregnant mares revealed the presence of a number of C_{19} steroids, including androstane-3,17-diol (2 isomers), androstane-3,16-diol, and 5-androstene-3,17-diol (2 isomers) [16]. In addition, oestradien-3,17-diol and oestratriene-3,17-diol were present and subsequently identified, again by GC–MS [16] (Fig. 1). HPLC was effective in purification to provide a fraction (F 22), which contained a mixture of only the oestradienediol and oestratrienediol (Fig. 1). Similar initial chromatography of allantoic fluid extracts yielded fractions, one of which contained oestrene-3,17-diol (3 isomers), oestradiene-3,17-diol (2 isomers) and oestratriene-3,17-diol (2 isomers) (Fig. 1). Argentation TLC of the purified allantoic fluid extracts yielded 2 fractions, F4 and F5, which contained, respectively, oestratrienediol, and (mostly) oestradienediol plus a smaller quantity of the trienediol (Fig. 1).

3.1. Investigation of the position of double bonds in the B ring of oestradienediol

The fractions obtained above, were then subjected to a series of spectroscopic, chemical and biochemical techniques:

3.1.1. Using UV spectroscopy

It is known that a conjugated 5,7- diene system, as in the case of our reference steroid, 7-dehydrocholesterol, exhibits absorbance maxima at 272, 282 and 292 nm (Fig. 2). Analysis of F22 by scanning UV spectroscopy showed an absorbance maximum at around 227 nm but there were no characteristic conjugated absorbance patterns (Fig. 2). The gradual decrease in absorbance from 227 to 327 nm may have been due to impurities in the sample.

3.1.2. Using hydrogenation

Portions of F22 were subjected to hydrogenation for 15, 30 or 45 min. In each case, GC–MS analysis showed the quantitative conversion of oestradienediol to one isomer of oestranediol (peak 1) and three mono-unsaturated products (peaks 2–4; Fig. 3). When the experiment was performed for 60 min, one of the oestrenediol isomers (peak 2) underwent further reduction to produce oestranediol (peak 1) leaving two mono-unsaturated structures, which were resistant to the effects of hydrogenation. The mono-unsaturated compound which proved to be facile to further reduction displayed a mass spectrum comparable to that of isomeric 5(10)-oestrenediol (see Table 1 for mass spectral characteristics).

Authentic reference material 7-dehydrocholesterol was hydrogenated under similar hydrogenation conditions, for 15, 30 or 45 min. The 2 mono-unsaturated products (Fig. 4) displayed mass spectra, with no peak at m/z 129, generated by cleavage through C-3 and C-6, and normally characteristic of TMS ether derivatives of 3-hydroxy 5-ene-steroids.

3.1.3. Using NMR analysis

Fraction 4 derived from allantoic fluid and containing presumptively, oestratrienediol, was studied initially. The spectrum was acquired at 400 MHz in $CDCl_3$ at room temperature and has been line-broadened to improve the signal to noise ratio (Fig. 5).

Although there appeared to be a few impurities, the spectrum represented essentially a single compound. No olefinic signals were seen; the 2H singlet at 6.89 ppm corresponds to the protons at the C-6 and C-7 positions in the aromatic B-ring, which degenerate in shift. The broad multiplet (4.13 and 3.93 ppm) presumably derives from the 3 proton and the sharp doublet (3.93 ppm) from the 17 proton. Both display downfield shift by the hydroxyl group. Chemical shift degeneracy in the rest of the spectrum precluded assignment of any other proton resonances. Although the recently published NMR data for the synthetic oestradienediols are incomplete [6], the available results are consistent with our present data.

The 1D NMR spectrum of fraction 5 (derived from allantoic fluid) and presumptively containing oestradienediol was acquired at 250 MHz on the same day as that of fraction 4, above. Approximately 35% of the sample was the aromatised triene structure (singlet and doublet peak at 6.89 and 3.93 ppm, respectively). A broad signal at 5.25 ppm was in the correct region for an olefinic proton and appeared to integrate as one proton, although the pulsing conditions for this experiment make these data unreliable. The 3 and 17 protons of the diol are well resolved at 4.08 and 3.79 ppm. This sample was re-run 10 days later, with a 10 s delay applied between transients in order to allow the systems to relax fully back to equilibrium. Under these condi-

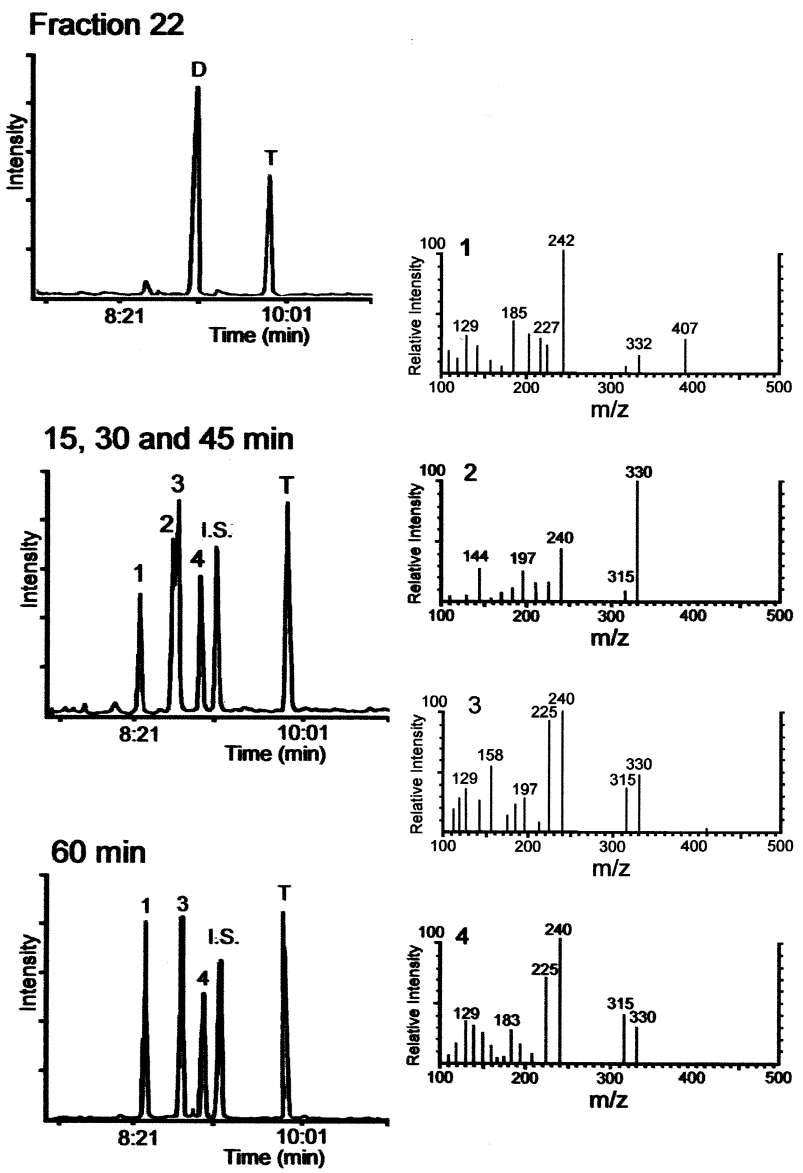


Fig. 3. Chemical hydrogenation of Fraction 22 obtained as in legends to Figs. 1 and 4. Portions of Fraction 22 was hydrogenated chemically (see Section 2) for 15, 30 and 45 min. Analysis of products was achieved using GC–MS, the relevant spectra being shown on the right: 1, oestranediol (1 isomer); 2, oestrenediol (3 isomers). After 60 min. hydrogenation, one isomer of peak 2 was converted into peak 1, the others were resistant. I.S., internal standard.

Table 1
Principal mass spectral ions of authentic and isolated C_{18} neutral steroids (as bis TMS ethers)[a]

Steroid	Standard	Isolated
Isomeric 5α-oestranediols	422 ($M^{+\bullet}$), 407, 332, 242.	407, 332, 242.
Isomeric 5(10)-oestrenediols	420 ($M^{+\bullet}$), 405, 330, 240.	420 (trace), 405, 330, 240.
5(10),7-oestradienediol	418 ($M^{+\bullet}$), 328, 238, 223 (100).	418 ($M^{+\bullet}$), 328, 238, 223.
5(10),7,9-oestratrienediol	416 ($M^{+\bullet}$), 401, 326, 311, 236 221.	416 ($M^{+\bullet}$), 401, 326, 311, 221.

[a] The base peak in each case is underlined. The series of C_{18} neutral steroids was isolated from equine pregnancy urine and allantoic fluid (see Section 2 and Fig. 1) or after microhydrogenation of 5(10),7-oestradienediol (see Fig. 3).

tions, integration was considered to be more reliable. The signal at 5.25 ppm, integrated for 1H, was indicative of an unconjugated diene structure. In contrast, the ^{1}H NMR spectrum of the reference compound (7-dehydrocholesterol) shows that the 6 and 7 olefinic protons appear quite distinctly resolved at 5.39 (H7) and 5.45 (H6), respectively (Fig. 5).

3.2. Instability of oestradienediol

It soon became apparent that the oestradienediol was extremely facile to oxidation, yielding the more chemically-stable ring B unsaturate, oestratrienediol. This phenomenon was exacerbated when derivatisation was performed at elevated temperatures; the conversion to oestratrienediol was minimised by derivatising samples at room temperature (Fig. 6).

Further, during our NMR studies, it was noted that the relative proportion of the triene- (3.93 ppm) to the dienediol (3.79 ppm) in fraction 4 (derived from allantoic fluid) had increased from 40 to 50% when the sample was re-analysed by NMR after storage for 10 days at 4°C in the dark under nitrogen (Fig. 5). After 4 weeks of storage under these conditions, no signal corresponding to the dienediol structure was observed. As in the prolonged storage of F 22, only the trienediol was detected when fraction 5 from allantoic fluid was re-analysed by GC–MS.

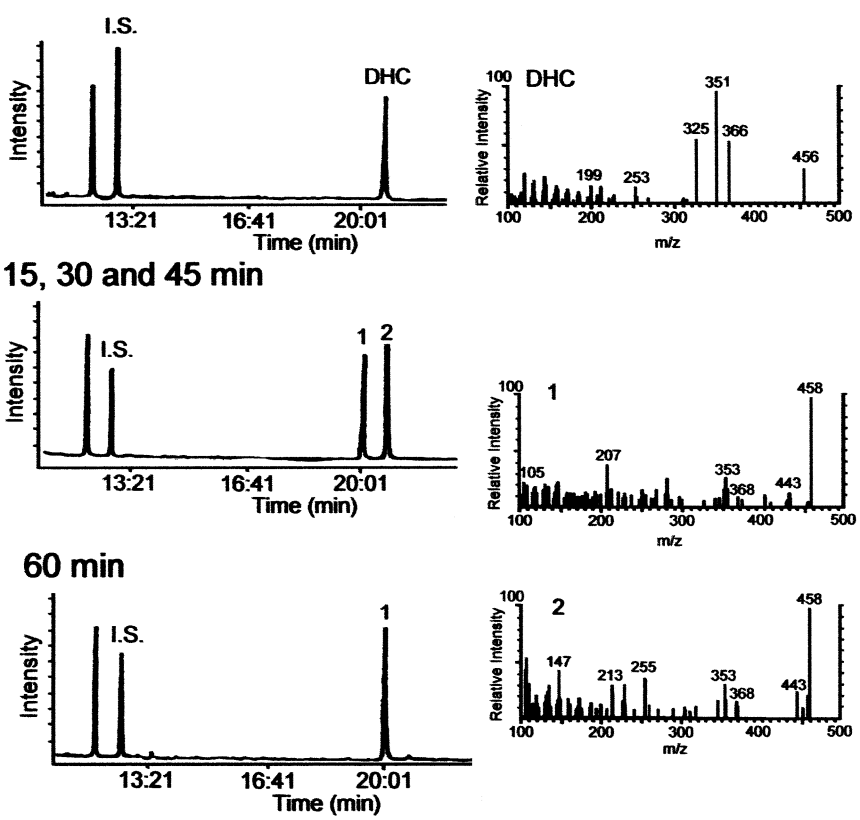


Fig. 4. Chemical hydrogenation of 7-dehydrocholesterol (DHC). The reference steroid, DHC, was subjected to hydrogenation under the conditions described in Section 2 and Legend to Fig. 3. Analysis of starting material and hydrogenation products was achieved by GC–MS, spectra being shown on the right: 1 and 2, 7-unsaturated mono-hydroxy-products. I.S., internal standard.

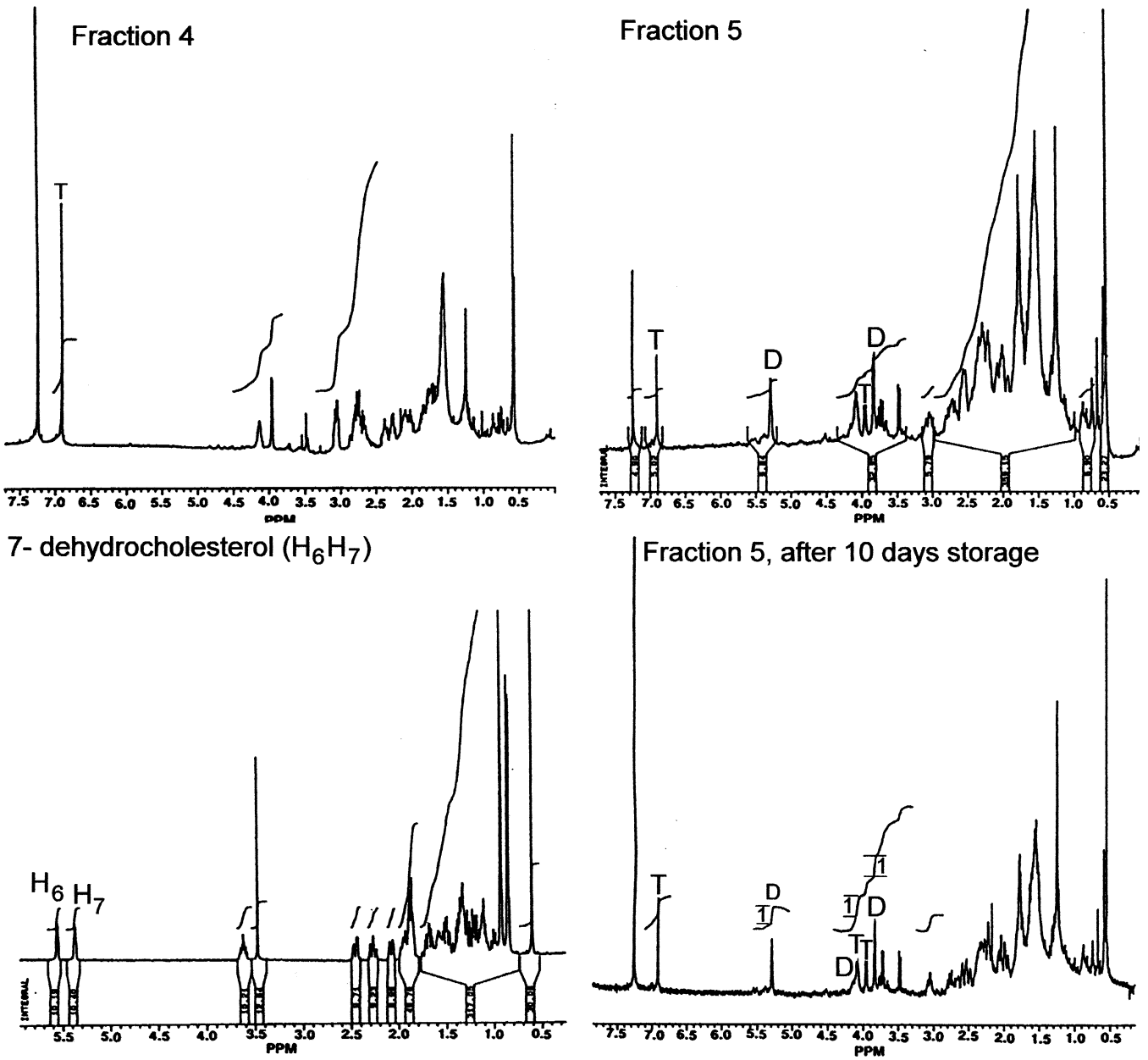


Fig. 5. NMR analysis of Fractions 4 and 5 and reference steroid 7-dehydrocholesterol. Fractions 4 and 5, obtained from equine allantoic fluid as described in Section 2 and Fig. 1, were subjected to NMR (see Section 2). Fraction 4 contained only oestratrienediol (T) while Fraction 5 contained both T and oestradienediol (D). After storage of Fraction 5 for 10 days at 4°C in the dark, the proportion of T to D increased from 40 to 50%, consistent with the facile oxidation of D to T. NMR of 7-dehydrocholesterol was performed to illustrate features in the spectrum characteristic of a conjugated 5,7-diene system (characteristics absent from the spectrum of D).

4. Discussion

4.1. Characterisation of oestradienediol

The main purpose of these studies was to isolate from pregnant mare urine and allantoic fluid large quantities of oestradienediol and oestratrienediol for subsequent characterisation. Fig. 1 shows that the chromatographic techniques we have employed have achieved this goal. Argentation TLC proved to be an extremely efficient way of separating the di-unsaturated from the tri-unsaturated C_{18} diol, a use noted in earlier studies for other steroids which only differed by one double bond [14]. This technique also exhibited a much higher capacity to process large quantities of these two steroids for further study than by HPLC.

The fractions isolated from purification of urinary and allantoic fluid extracts (F22 and F4, F5) respectively were subjected to UV and NMR analysis and various processes, including hydrogenation and treat-

ment with 5-ene-3β-HSD-isomerase. The results of these studies are summarised below:

- the oestradienediol in the fractions was readily oxidised to the ring-B aromatic diol oestra-5,7,9(10)-triene-3,17-diol;
- UV spectroscopy showed that F22 did not contain a conjugated diene;
- NMR analysis of F5 showed the presence of a single olefinic proton;
- hydrogenation of F22 produced isomers of 5(10)-oestrene-3,17-diol and oestrane-3,17-diol; and
- the unsaturated steroids in the fractions and the reference steroid 5(10)-oestrene-3,17-diol were resistant to isomerisation by the enzyme 5-ene-3β-HSD-isomerase.

The facile oxidation of oestradienediol to oestratrienediol, which we noticed incidentally in the derivatisation procedures (Fig. 6), indicates (1) that both compounds are structurally related, i.e. retain similar 3 and 17 configuration; and (2) that the sites of unsaturation both reside in the B-ring of the steroid nucleus (as discussed above). It is worth noting that this oxidative conversion explains the anomaly between argentation TLC being an effective technique in separating the oestradienediol from the oestratrienediol but GC–MS analysis always indicating a proportion of the oestratrienediol contaminating the oestradienediol chromatogram (Fig. 1). It is also worth noting that another group has found that the isomeric 5(10),7-oestradienediols were stable in synthetic studies [7]. However, virtually all the chemical steps used in synthesis were performed at room temperature, in contrast to most of our own experiments, and this may help to explain the seeming discrepancy between the results.

For the oestradienediol to possess an unconjugated diene system as indicated by UV, it would need to have sites of unsaturation in ring-B at C-5(10),7-, C-5,8- or C-6,9(10)-. NMR (Fig. 5) showed the presence of only a single olefinic proton which eliminates the possibility of unsaturation at C6,9(10).

Of the remaining possible sites of unsaturation, C-5(10),7- and C-5,8-, the hydrogenation results favour the former. Hydrogenation of the purified urinary extract F22 and analysis of the silylated extract by GC–MS yielded a complex mixture (Fig. 1) containing silyl derivatives of oestrane-3,17-diol and three mono-unsaturated diols. The mass spectrum of one of the silylated mono-unsaturated diols was comparable to that of authentic derivatised 5(10)-oestrene-3,17-diol (Table 1), supporting a site of unsaturation at C-5(10)-.

Hydrogenation of 7-dehydrocholesterol (Fig. 4) showed facile reduction at C-5 to produce two mono-unsaturated products presumably 5α and 5β. If the data generated for the hydrogenation pattern of 7-dehydrocholesterol were consistent with other 5-ene systems e.g. C-5, 8-, the latter would yield two products produced from reduction at C-5, particularly as the site of unsaturation at C-8 is probably more sterically hindered to hydrogenation than the C-7 site of 7-dehydrocholesterol. This is not consistent with the complex hydrogenation pattern produced by F22 (Fig. 3) and thus again favours sites of unsaturation at C-5(10),7-.

Further support for the C-5(10),7- structure for oestradiene-3,17-diol was obtained from in vitro incubation studies with 5-ene-3β-HSD-isomerase. This enzyme catalyses the isomerisation of 5-ene-3β-hydroxy steroids to the corresponding 4-en-3-oxo configuration.

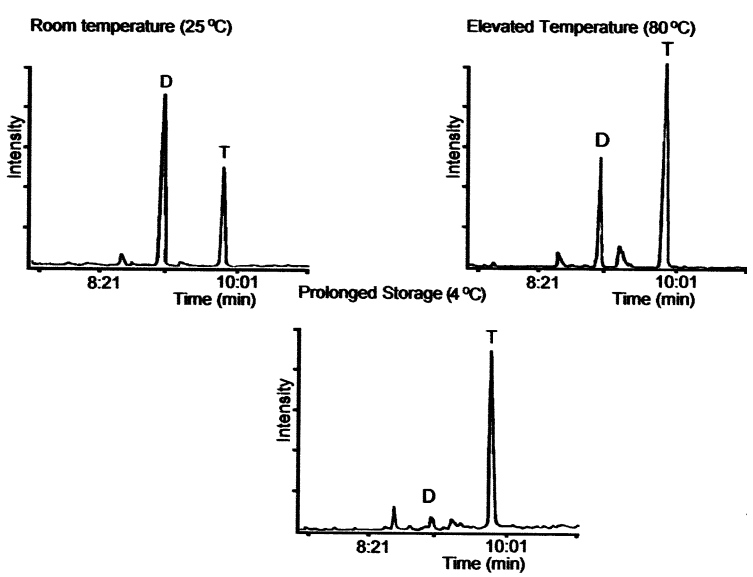


Fig. 6. Effect on the oxidation of oestradienediol of conditions used for conversion to its bis trimethylsilyl (TMS) ether. Portions of oestradienediol (D) (obtained and described as in Fig. 1) were derivatised (see Section 2) at 25°C and 80°C. The bis TMS ether reaction mixture performed at 25°C was stored at 4°C for 10 days. In each case, reaction products were analysed by GC–MS.

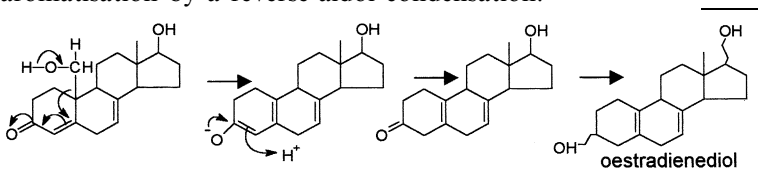
If the oestradienediol were to contain a 5-ene bond, exposure to the enzyme would be expected to result in the formation of mono-unsaturated analogues of 19-nortestosterone. However, the purified fractions containing oestradienediol and oestratrienediol and authentic standard 5(10)-oestrenediol proved resistant to the effects of this enzyme leaving only the starting material detectable, as shown by GC–MS analysis (results not shown). Under the same conditions, however, DHA (a 5-ene-3β-hydroxysteroid) could be regularly converted into 4-androstenedione (principal mass spectral ions of MO derivative at m/z 344 ($M^{+\bullet}$), 313, 267, 225, 153) (cf. ref. 4). This clearly shows that the enzyme was active.

Finally, support of the C-5(10),7-diene structure was obtained from NMR analysis. The NMR spectrum (Fig. 5) generated for the purified fraction (F5) from allantoic fluid which contained mainly oestradienediol, showed a broad signal at 5.25 ppm which integrated for 1H. This is consistent with an unconjugated C-5(10),7-diene structure for the oestradienediol.

4.2. Heard's ketone an artefact?

From our present results, 5(10),7-oestradienediol may be considered as the precursor of oestratrienediol, i.e. the diol of Heard's ketone. If the latter is an artefact of the isolation and identification procedures adopted in our work and previous studies [8] then, by definition, Heard's ketone, 3-hydroxy-5(10),6,8-oestratrien-17-one, may well be considered an artefact of the related C_{18} di-unsaturated mono-hydroxy monoketonic compound. Heard's ketone has been detected in in vitro incubation studies using placental preparations together with 3β-hydroxy-5,7-andostadien-17-one or 3-hydroxy-3,5,7-oestratrien-17-one [17]. We suggest that a 5,7-oestradiene mono hydroxy-ketonic structure is the true metabolite in these studies and that Heard's ketone subsequently arises as an artefact of the isolation procedures [8].

Heard's ketone and 5(10)-oestrenediol have been shown to be metabolites from suitable C_{19} compounds in in vitro incubation studies using equine placental and testicular tissues [17,18]. On the basis of this work [17,18] and of other studies [19], it is feasible that oestadienediol may arise as a result of demethylation without concomitant A-ring aromatisation of suitable C_{19} steroids. Essentially, cleavage of the C-19 methyl group occurs but the A-ring becomes stabilised against aromatisation by a reverse-aldol condensation:



The reduction can either occur at C-10, yielding a 4-ene structure such as nortestosterone, or at C-4 resulting in a 5(10)-ene norandrogen [19]. Nortestosterone has been shown to act as a substrate for aromatisation in human and equine placental preparations [20,21] while the isomerisation of 5(10)-oestrenediol to nortestosterone has been displayed in equine testicular preparations [17,18]. It has been demonstrated from in situ cannulation studies of equine umbilical cord that 5(10)-oestrenediol is formed in preference to nortestosterone at the placental surface [4]. Demethylation of C_{19} steroids by tissues displaying intrinsically high aromatising capabilities [22] may be proposed as a physiological mechanism for controlling oestrogen production and thus circulatory levels in the animal.

The possible biological significance of the C_{18} diols, including oestradienediol, has been noted earlier in this paper [5,6], and the oestrogenicity of the dienediol [7] seems to be consistent with these findings. It is hoped that further work may elucidate the role of C_{18} diols in equine pregnancy.

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