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Determination of Oestrone, 17α- and 17β-Oestradiol in Bovine Aqueous Humor Using Gas Chromatography-Negative Ion Chemical Ionization Mass Spectrometry

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Perfluorotolyl (PFT)-ether and perfluorotoly-trimethylsilyl (PFT-TMS) ether derivatives of oestrone, 17α- and 17β-oestradiol were prepared under phase transfer conditons. The former derivatives under negative ion chemical ionization conditions gave significant ions in the mass spectrometer but 17α- and 17β-oestradiol gave poor resolution. However, the PFT-TMS derivatives of 17α- and 17β-oestradiol showed good resolution. These derivatives were used for the analysis of oestrogens in bovine aqueous humour, vitreous humour and retina. The mean (±SEM) concentrations of oestrone in bovine aqueous humour (n=18), vitreous humour (n=18) and bovine retina (n=4) were 0.47±0.11, 0.46±0.14 and 1.10±0.24 ng.ml^{-1}, respectively. 17α-Oestradiol was detected in 16 out of 18 samples of bovine aqueous humour and vitreous humour and the mean (±SEM) concentrations were 0.30±0.10 and 0.08±0.02 ng.ml^{-1}, respectively. The mean (±SEM) concentration of 17β-oestradiol in aqueous humour (n=7) and vitreous homour (n=11) 0.83±0.26 ng ml^{-1} and 0.39±0.09 ng ml^{-1}, respectively. In retina the concentrations of both steroids were below the detection limit.

Key words: Oestrone, 17α-Oestradiol, 17β-Oestradial, GC-MS, Aqueous humour, Vitreous humour, Retina

INTRODUCTION

The intraocular pressure (IOP) is higher in women than that of men (Graham and Hallows, 1964: Said-U-Zafar and Vaid, 1968), and a rise in IOP was also observed during menstruation (Vaid *et al.*, 1965). The IOP is relatively high during the secretory phase, followed by a decrease during the ovulatory phase and then, again increases during the luteal phase. The concentration of oestrogens in the menstrual cycle is high during the secretory phase and then drops suddenly during the ovulation phase, followed by a slight increase during the luteal phase. Thus, the increase in IOP may be related to the concentration of oestrogens (Vaid *el al.*, 1965). The exact role of oestrogens in regulating the IOP is not clear but it has been found that the activity of hyaluronidase, an enzyme responsible for the depolymerization of hyaluronic acid, is inhibited by the follicular hormones (oestrogens) and enhanced by the corpus luteum hormones (progesterone and relaxin) (Obal, 1950). Thus, it is possible that steroidal hormones may play an important role in lowering the IOP by depolymerisation of hyaluronic acid in the trabecular meshwork.

Various methods has been used for the analysis of oestrogens. Radioimmunoassay has been used for the analysis of the oestrogens in aqueous humour but it was not possible to report the individual oestrogens using this technique (Starka *et al.*, 1976). These immunological methods are limited due to the non-specificity of antibodies and the use of radioisotopes.

The limit of detection of MO-TMS derivative of oestrone and diTMS the derivative of 17β-oestradiol under EI GC-MS was 1 ng and 100 pg respectively (Ichimura, *et al.*, 1986). These derivatives were used to study the *in vitro* biosynthesis of oestrogens in human foetal liver (Cantineau, *et al.*, 1985).

Derivatization of 17β-oestradiol and oestrone with 3,5-Bis(trifluoromethyl) benzoyl chloride (TFMBO) formed the di-TFMBO as a major and TFMBO as a minor derivative of 17β-oestradial while, TFMBO-oestrone derivative of oestrone (Murray and Watson, 1986). The main disadvantage of this method was the formation of a mixture derivatives which may not be suitable for the analysis of these steroids at very low concentration using GC-NICIMS under SIM.

The aim of the present work is to develop a suitable gas chromatographic-negativeion chemical ionization mass spectrometric (GC-NICIMS) method for

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the analysis of the oestrogens in bovine aqueous humour, vitreous humour and retina, which are used as a model eye. The study of endogenous steroids in the eye may help to understand the possible role of the steroids in maintaining the IOP in general and in open angle glaucoma in particular.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: Octafluorotoluene (OFT), N, O-bis(trimethylsily) acetamide (BSA), deuterioethanol, deuterium oxide, deuterium chloride (Aldrich Chem. Co., Dorset, UK.); anhydrous sodium sulphate, sodium hydroxide, sodium hydrogen sulphate, potassium tert but oxide, hydrochloric acid and acetic acid (BDH-Merck, Poole, Dorset, UK.); ethyl acetate, hexane, water and dichloromethane (HPLC grade, Rathburn Chemical Co., Walkerburn, Peebleshire, UK.); oestrone, 17α-oestradionl, 17β-oestradiol, and Sephadex LH-20 (Sigma Chemical Co., Ltd., Dorset, UK.).

Preparation of [$^2H_2$] 17β-Oestradiol and [$^2H_2$] Oestrone

Oestrone or 17β-oestradiol (100 mg) was dissolved in a solution of potassium tert butoxide (3 ml; 0.05 M in $C_2H_5O^2H$, w/v) and heated in a sealed tube at 80°C, for 4h; for the synthesis of labelled 17β-oestradiol and, for 24h; for the synthesis of deuterated oestrone. ^2HCl (0.5 ml; 35%, w/v) was then added, and the sample diluted with ^2H_2O (5 ml) and extracted with ethyl acetate (2×5 ml). The organic layer was dried over anhydrous sodium sulphate and the solvent was removed by evaporation under the stream of nitrogen. The residues were redissolved in small amount of ethyl acetate and crystallization was induced by the addition of hexane.

17α-Oestradiol under similar conditions failed to give the deuterated product. The deuterated isotopomers of oestrone and 17β-oestradiol were yellow crystalline solids.

The relative amount of different deuterated species in the products was determined by preparing the PFT and PFT-TMS derivatives of deuterated oestrone and 17β-oestradiol, respectively. These derivatives were then injected into GC-MS under the negative ion chemical ionization (NICI) mode with selected ion monitoring (SIM) of the ions at *m/z* 270, 269 and 268 for oestrone and 344, 343 and 342 for 17β-oestradiol.

The [$^2H_2$] oestrone and [$^2H_2$] 17β-oestradiol were used without further purification as an internal standard.

Formation of Perfluorotoly (PFT) and Perfluorotolytrimethylsilyl (PFT-TMS) Derivatives of Standard Oestrogens

PFT derivatives of oestrone, 17α- and 17β-oestradiol and for the mixture of these oestrogens were prepared as follows: OFT (50 µl), aqueous sodium hydroxide (1 ml; 1 M) and tetra-n-butylammonium hydrogen sulphate (1 ml; 0.1 M) were added to the solution of steroid (10 µg) in dichloromethane (1 ml). The two phase system was shaken at room temperature for 2h and centrifuged. The aqueous layer was discarded and organic layer was then evaporated under a stream of nitrogen. The residue was redissolved in hexane (1 ml), water (1 ml; HPLC grade) was added and mixture was shaken for 5 min. The organic layer was separated and dried by passing it through a short column of anhydrous sodium sulphate (ca≈3 cm, in Pasteur pipette). The hexane elute was evaporated under a stream of nitrogen, the residue was dissolved in ethyl acetate (40 µl) and 2 µl was injected into the GC-MS for analysis.

PFT-TMS derivatives were prepared by treating the residue obtained after the evaporation of the hexane, with BSA (40 µl, 60°C, 30 min). Excess of reagent was then evaporated under a stream of nitrogen, the residue was redissolved in ethyl acetate (40 µl) and 2 µl was injected into the GC-MS for analysis.

Gas Chromatography-Mass Spectrometry

GC-MS analysis was carried out using a Hewlett-Packard 5988A GC-MS, interfaced with a HP-RTE 6/VM data system. Analysis was carried out under negative ion chemical ionization (NICI) mode. Methane was used as a reagent gas with a source pressure of ≈ 1 Torr and electron energy was set at 200 eV. The instrument was tuned to the ions at *m/z* 452, 595 and 633 of the perfluorotributylamine (PFTBA) calibrant. Source temperature was adjusted at 140°C.

The mass spectrometer was coupled to a Hewlett-Packard 5890 gas chromatograph with a transfer line temperature of 280°C. For all analyses the GC was fitted with a Restek Rtx-1 (cross linked methyl silicone (capillary column (12 m×0.25 mm I.D.; 0.25 µm film thickness). The following GC conditions were held constant: the injector temperature was 250°C and helium carrier gas was used with a head pressure of 5 p.s.i. The temperature programme conditions used in the analysis of PFT-TMS derivatives of oestrogens was; 190°C (1 min), 20°C min^{-1}, 260°C 15 (min) and for PFT derivatives of oestrogens was; 190°C (1 min), 20°C min^{-1}, 260°C (10 min).

Biological Samples

Eyes from freshly killed cattle were obtained from a local abattoir. Aqueous and vitreous humour and retina were collected within one hour of death of the animal.

Aqueous humour (ca 1 ml) was collected with a

Pasteur pipette from the anterior chamber after excising the cornea and samples were stored in a vials with screw caps at -20°C.

Vitreous humour was collected after removing the aqueous humour, cornea and lens. The samples (4 to 5 ml) were stored in small jars with screw tops at -20°C.

Retina was obtained by carefully detaching it from the eye with a microspatula, after the removal of the aqueous humour, cornea, lens and vitreous humour. It was wrapped in aluminum foil and stored at -20°C.

Extraction and Derivatization of Endogenous Steroids

Aqueous Humour: Prior to analysis aqueous humour was defrosted at room temperature and then an accurately measured volume (1×5 ml) was transferred to a test tube with a screw cap (10 ml). Then an aliquot of a standard solution (5 µl; 1 ng μl^{-1} in acetonitrile) of deuterated oestrone and deuterated 17β-oestradiol was added. The sample was then shaken for 5 min to disperse the standards.

The aqueous layer was extracted with ethyl acetate (2×5 ml) and the combined organic layers were dried by passing through a short column of anhydrous sodium sulphate (ca 3 cm in a Pasteur pipette). The ethyl acetate was evaporated under the stream of nitrogen and the residue was converted to PFT-TMS derivatives for the analysis of oestrone, 17α-oestradiol and 17β-oestradiol. The final volume of the derivatized sample was 2 µl and the whole sample was injected into the GC-MS for analysis.

Vitreous Humour: Vitreous humour was extracted and derivatized in a manner identical to that described for aqueous humour.

Retina: The retina was defrosted, weighed and transferred to a glass homogenizer. Then an aliquot (5 µl) of standard solution (1 ng μl^{-1} in acetonitrile) of deuterated oestrone and deuterated 17β-oestradiol was added and the retina was homogenized with water (0.25 ml) for 5 min, then hexane (0.5 ml) was added and sample was further homogenized for 10 min. Organic layer was collected and sample was again extracted with hexane (1.5 ml). The combined hexane fractions were dried over anhydrous sodium sulphate and solvant was evaporated under a stream of nitrogen. The residue was derivatized to PFT-TMS derivatives and injected into GC-MS in the similar way as discussed in aqueous humour.

RESULTS AND DISCUSSION

Quantification of Oestrogens

It was reported earlier that octafluorotoluene (OFT) reacts rapidly under phase transfer conditions with phenolic hydroxyl groups to form perfluorotolyl (PFT) ether derivatives and that reaction with the phenolic hydroxyl at C3 of 17β-oestradiol at room temperature was completed in 1h (Jarman, 1985). Thus PFT derivatives of oestrone, 17α- and 17β-oestradiol were prepared under phase-transfer conditions. Reaction for 1h gave a poor yield of the derivatives but shaking the reaction mixture for 2h improved the yield of the PFT derivatives.

Table I. Mass spectral data for derivatives of oestrogens under NICI conditions

Oestrogen	Derivative	M^- *m/z*	Other Significant Longs (*m/z*)
Oestrone	PFT	486 (24%)	268 (100%), 217 (63.8%)
17α-Oestradiol	PFT	488 (20%)	217 (100%), 270 (48.6%)
	PFT-TMS	560 (15%)	217 (100%), 342 (56.6%)
17βOestradiol	PFT	488 (19%)	217 (100%), 270 (49.1%)
	PFT-TMS	560 (19%)	342 (100%), 217 (91.7)

The mass spectrum of the PFT derivative of oestrone under NICI conditions gave a base peak at *m/z* 268 (M^--PFT), the molecular ion peak was at *m/z* 486 (M^-; 23.5%) and the reagent ion $(C_7F_7)^-$ specific peak at *m/z* 217 (63.8%). The mass spectral data for oestrone, 17α- and 17β-oestradiol are summarised in Table I.

The yield of PFT derivatives of oestrogens was good and characteristic ions were obtained in high abundance but the retention times of the PFT-derivatives of 17α- and 17β-oestradial were very similar and it was not possible to resolve the two peaks completely. A mixed derivative was prepared with a PFT ether at the 3 position and a TMS ether at the 17 position. The mass spectra of the PFT-TMS derivatives of 17α- and 17β-oestradiol obtanied under NICI conditions are shown in Fig. 1 and the mass spectral data of these derivatives are summarized in Table I.

The principal ions in the mass spectrum of the PFT-TMS derivatives were as follows: 17α-oestradiol, *m/z* 560 (M^-, 15.4%), *m/z* 342 (M^--PFTH, 57.6%) and *m/z* 217 (reagent specific ion, 100%) and for the 17β-oestradiol PFT-TMS derivative the ions were as follows: *m/z* 560 (M^--, 19.3%), *m/z* 342 (M^--PFTH, 100%) and *m/z* 217 (reagent specific ion, 91.7%). The ions selected for SIM and the limit of detection for the PFT and PFT-TMS derivatives of oestrone, 17α- and 17β-oestradiol are shown in Table II.

Mass Spectral Data for Deuterated Internal Standards

Comparison of the mass spectra obtained for the PFT derivatives of both labelled and unlabelled oestrone (see Fig. 2) showed that: $[^2H_2]$-oestrone was the

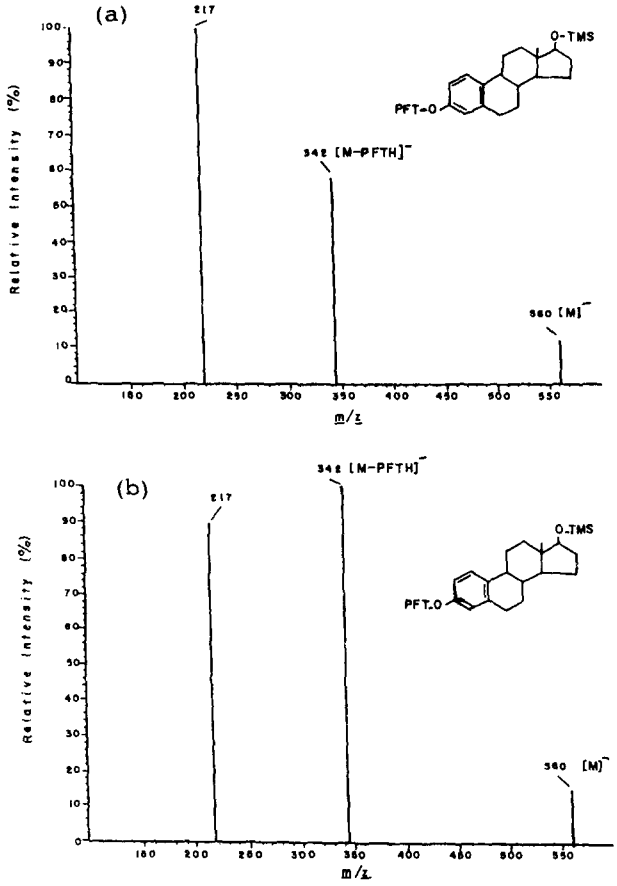

Fig. 1. The NICI mass specta of PFT-TMS derivatives of a) 17α-oestradiol, b) 17β-oestradiol.

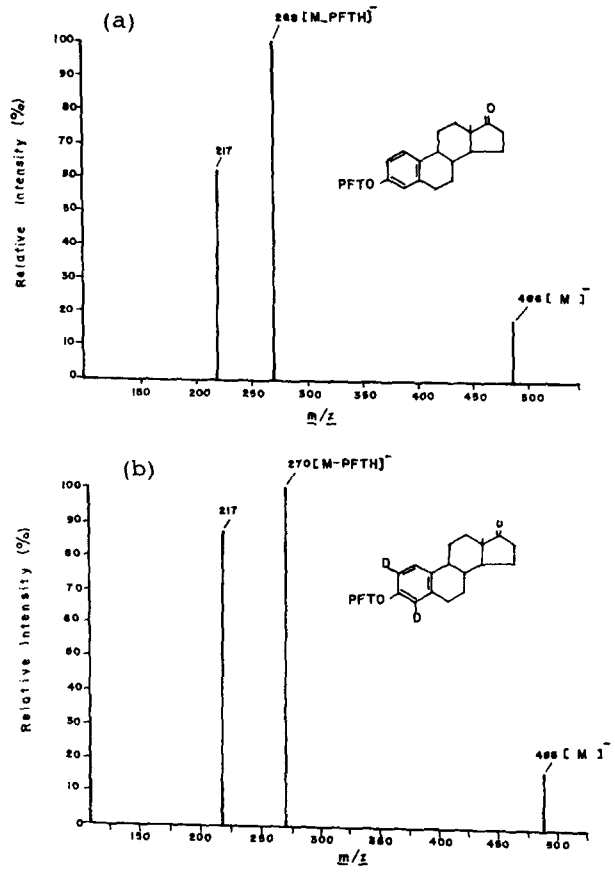

Fig. 2. The NICI mass spectra of PFT derivatives of a) oestrone, b) [D_2] oestrone.

Table II. Ion selected for SIM and practical limit of detection of PFT-TMS derivatives of oestrogens

*Oestrogen	Ion Selected for SIM (*m/z*)	Limit of Detection (pg)
Oestrone	268	30
17α-Oestradiol	342	30
17β-Oestradiol	342	10

major product, i.e. 84.1%, [$^2H_1$] was 15.8% the amount of the [$^2H_0$] isotopomer was < 0.1% and the two hydrogen atoms replaced by deuterium were presumably at the 2 and 4 positions. The GC-NICIMS spectra of both labelled and unlabelled 17β-oestradiol showed that the [$^2H_2$] 17β-oestradiol was the major product (95.9%), the amount of the [$^2H_1$] and [$^2H_0$] isotopomer were 3.8% and 0.3%, respectively and the two hydrogen atoms replaced by deuterium were presumably at the 2 and 4 positions.

The labelled oestrogens thus obtained were suitable for use as internal standards, particularly [$^2H_2$] oestrone with < 0.1% of the [$^2H_0$] product. The amount of [$^2H_0$] product in the labelled 17β-oestradiol was within the acceptable limits for use as an internal standard.

Calibration Curves

The GC-MS response was calibrated by analysis of varying amounts of derivatized unlabelled oestrogen (0.5 to 10.0 ng ml^{-1}) against fixed amounts (5 ng) of the corresponding derivatized deuterium-labelled steroid. The plots for the area of the peaks for oestrone, 17α- and 17β-oestradiol against the appropriate internal standards were linear over the range of 0.5 to 10.0 ng ml^{-1}.

Quantification of Oestrogens in Bovine Eye

Aqueous Humour: Bovine aqueous humour samples (n=18) were analyzed simultaneously for the presence of oestrone, 17α- and 17β-oestradiol. Before analyzing the biological samples a blank sample (containing only deuterated standards; 5 ng of each) was analyzed in order to eliminate the possibility of any contamination of the standards and solvents. In addition, a standard 1:1 mixture of derivatized labelled and unlabelled standard oestrogens (5 ng each) was analyzed at the end of each batch of biological samples to allow for variations in the instrumental response.

Bovine aqueous humour (n=18) showed the presence of oestrone in all samples with a mean (±SEM) concentration of 0.473 (±0.105 ng ml^{-1}), with a range of 0.04 to 1.40 ng ml^{-1}. 17α-Oestradiol was quantified in 16 samples of bovine aqueous humour with a mean (±SEM) concentration of 0.30 (±0.10 ng ml^{-1}), with a range of 0.01 to 1.15 ng ml^{-1}. Even with the very low detection limit for 17β-oestradiol (10 pg), it was quantified in only seven samples and in rest of the samples, its concentration was below this level. The mean (±SEM) concentration of 17β-oestradiol was 0.83 (±0.26 ng ml^{-1}), with a range of 0.100 to 2.08 ng ml^{-1}. Table III shows the concentratios for oestrone, 17α-oestradiol and 17β-oestradiol in aqueous humour and Fig. 3 shows the SIM traces for oestrone, 17α- and 17β-oestradiol extracted from bovine aqueous humour.

Table III. Mean concentration (ng ml^{-1}) of oestrone, 17α- and 17β-oestradiol in bovine aqueous humour, vitreos humour and retina

Oestrogen	Aqueous humour		Vitreous humour		Retina	
	n	Mean (±SEM)	n	Mean (±SEM)	n	Mean (±SEM)
oestrone	18	0.47 (0.11)	18	0.46 (0.14)	4	1,10 (0.24)
17α-oestradiol	16	0.30 (0.10)	16	0.08 (0.02)		-
17β-oestradiol	07	0.83 (0.26)	11	0.39 (0.09)		-

n=No. of samples analyzed for steroid.

Vitreous Humour: Eighteen (18) samples of bovine vitreous humour were analyzed for the presence of oestrone, 17α- and 17β-oestradiol. Oestrone was detected in all samples of bovine vitreous humour with a mean (±SEM) concentration of 0.46 (±0.14 ng ml^{-1}), with a range of 0.05 to 1.93 ng ml^{-1}. 17α-Oestradiol was detected in 16 samples of bovine vitreous humour with a mean (±SEM) concentration of 0.08 (±0.02

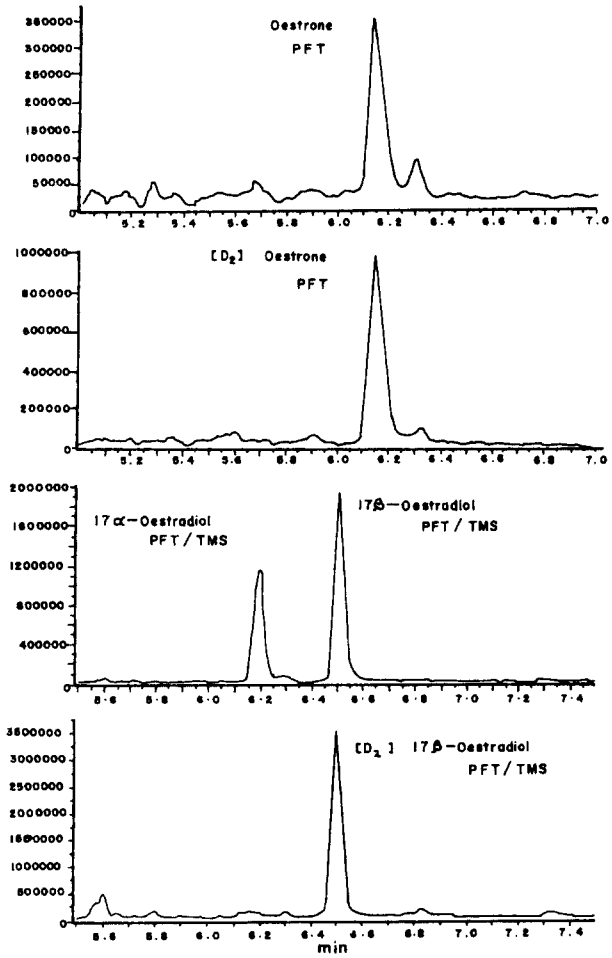

Fig. 3. SIM traces of endogenous steroids in bovine aqueous humour (4 ml), following the addition of corresponding deuterated internal standards (5 ng), extraction and formation of PFT-TMS derivatives.

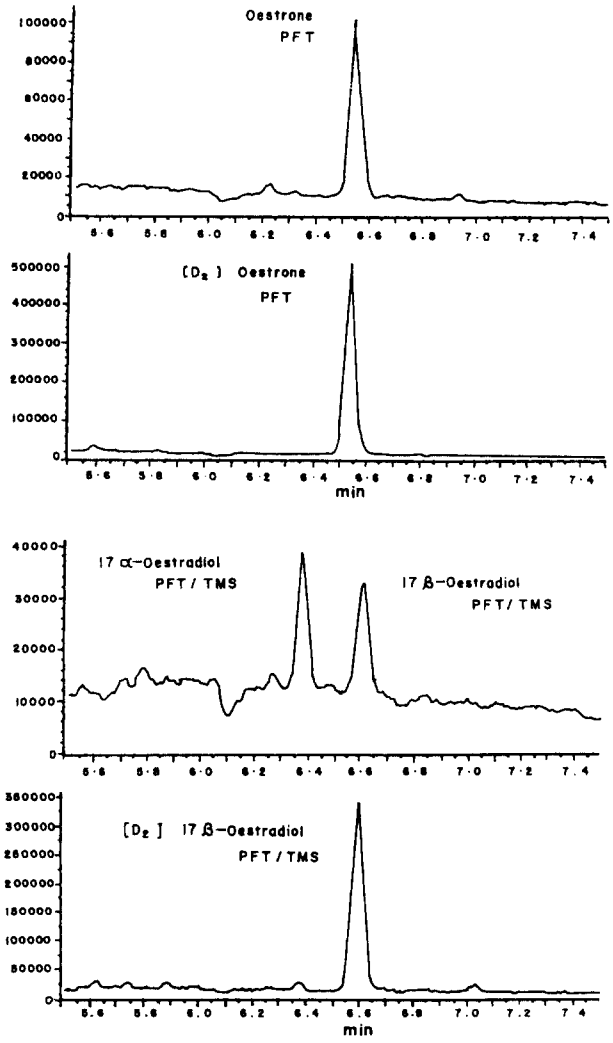

Fig. 4. SIM traces of endogenous steroids in vitreous humour (4 ml), following the addition of corresponding deuterated internal standards (5 ng), extraction and formation of PFT-TMS derivatives.

ng ml^{-1}), with a range of 0.02 to 0.25 ng ml^{-1} and 17β-oestradiol was quantified in 11 samples of bovine vitreous humour with a mean (±SEM) concentration of 0.39 (±0.09 ng ml^{-1}), with a range of 0.08 to 1.06 ng ml^{-1}. Table III lists the concentrations and Fig 4 shows the GC-NICIMS-SIM traces of oestrogens extracted from bovine vitreous humour.

Rethina: Four samples of bovine retina were analyzed for the presence of the oestrone, 17α- and 17β-oestradiol. Oestrone was present in all the samples of the bovine retina and its mean concentration (±SEM) was 1.10 (±0.24) ng ml^{-1} with a range of 0.66 to 1.77 ng g^{-1}. The concentration of oestrone was high as compared to the concentrations determined in bovine aqueous and vitreous humour, but, unlike aqueous and vitreous humour, the amounts of both 17α- and 17β-oestradiol were below the limit of detection.

The mean concentrations of oestrone, 17α- and 17β-oestradiol in bovine retina, aqueous and vitreous humour are shown in Table III.

It has been observed that the concentrations of oestrone in aqueous and vitreous humour are identical while its concentration is high in retina (see Table III). 17α-oestradiol was detected consistently, both in bovine aqueous and vitreous humour, where as 17β-oestradiol was not quantified in all samples. The presence of 17α- and 17β-oestradiol in bovine aqueous humour and vitreous humour may be due to the metabolism of oestrone by tissues adjacent to the aqueous humour in the anterior chamber of the eye (Starka, 1975).

The role of the endogenous steroids in eye can not be ignored in the regulation of IOP. Further studies on the analysis of these steroids both in male and female glaucomatous eye and non-glaucomatous eye will help to understand the mechanism of the regulation of IOP.

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