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The quantification of endogenous steroids in bovine aqueous humour and vitreous humour using isotope dilution GC-NCI-MS

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Abstract

Pentafluorobenzyloxime-trimethylsilyl derivatives of androgens, progestogens and corticosteroids were prepared and used for the analysis of these steroids in bovine aqueous humour and vitreous humour by GC-MS method. Appropriate deuteriated isotopomers of the parent steroids were labelled with deuterium via simple synthetic procedure and used as internal standards. The concentration (ng ml^{-1}, $\pm$S.E.M.) of these steroids in bovine aqueous humour and vitreous humour were found to be as follow: (1) aqueous humour ($n=17$): hydrocortisone ($n=17$; 2.40 ± 0.54), progesterone ($n=15$; 0.06 ± 0.01), 4-androstene-3,17-dione ($n=8$; 0.15 ± 0.07) and testosterone ($n=4$; 0.14 ± 0.04); and (2) bovine vitreous humour ($n=19$): hydrocortisone ($n=19$; 1.78 ± 0.25), progesterone ($n=18$; 0.09 ± 0.01), 4-androstene-3,17-dione ($n=19$; 0.11 ± 0.02), 11-deoxycorticosterone ($n=12$; 29.27 ± 6.42), 17α-hydroxyprogesterone ($n=6$; 5.55 ± 3.12). The concentration of corticosterone, 11-deoxycorticosterone and 17α-hydroxyprogesterone and testosterone and corticosterone were below the limit of detection in aqueous humour and vitreous humour, respectively.

Keywords: Aqueous humour; Vitreous humour; Steroids; GC-MS; Negative ion chemical ionization; Intraocular pressure

1. Introduction

The elevation of intraocular pressure (IOP) following the corticosteroid therapy for various eye diseases has been documented extensively [1–7]. In mice, a high IOP (38–42 mmHg) following the subcutaneous injection of testosterone propionate has been observed [8]. The topical application of progesterone [9] and corticosteroid inhibitor; RU-86-6 (1% solution) to the rabbit's eye, decreased the IOP [10]. The presence of abnormal metabolites of hydrocortisone (5α- and 5β-dihydrocortisol) in cultured TM cells [11] and in rabbits these metabolites potentiated a rise in IOP caused by dexamethasone. These studies indicate that endogenous steroids may play vital role in the maintenance of IOP and their abnormal concen-

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tration or metabolism may lead to the open angle glaucoma (OAG).

Various techniques have been used for the determination of steroids in aqueous humour; these include colourimetric [12,13], paper chromatographic [14], radioimmunoassay (RIA) [15,16] and GC-MS methods [17].

The present study was carried out to determine the endogenous steroids in bovine aqueous humour and vitreous humour using stable isotope dilution GC-negative ion chemical ionization (NICI-MS).

2. Experimental

2.1. Materials

Chemicals were obtained from the following sources: pentafluorobenzylhydroxylamine hydrochloride (PFBO HCl) (Aldrich Chem. Co., Dorset, UK); potassium *tert* butoxide, anhydrous sodium sulphate, (DTFMB), (BDH-Merck, Poole, Dorset, UK); trimethylsilyl-imidazole (TMS IM) (Fluka, Derbyshire, UK); ethyl acetate, hexane and water (HPLC grade, Rathburn Chemical Co., Walkerburn, Peebleshire, UK); hydrocortisone, testosterone, 4-androstene-3,17-dione, progesterone, 17α-hydroxyprogesterone, corticosterone and deoxycorticosterone, deuterioethanol, deuterium oxide, deuterium chloride and Sephadex LH-20 (Sigma Chemical Co. Ltd, Dorset, UK). The labeled internal standards were synthesized in the laboratory.

2.2. GC-MS

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

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 (25 m × 0.25 mm id; 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 program conditions used for the analysis of PFBO-TMS derivatives of steroids was; 190°C (1 min), 30°C min^{-1}, 320°C (15 min).

Table 1
Mass spectral data, diagnostic ions, retention time and limit of detection for PFBO-TMS derivatives of steroids under NICI conditions

Steroid	Base peak (m/z)	Ions used for SIM (m/z)	LOD (pg)[a]	Other significant ions (%) (m/z)	Rt (min)
Testosterone	535	535 $[M\text{-}HF]^-$	20	555 (1.0), 377 (21.8), 197 (7.1)	6.47
4-Androstene-3,17-dione	656	656 $[M\text{-}HF]^-$	10	676 (3.0), 495 (17.5), 196 (48.0)	10.50
Progesterone	684	684 $[M\text{-}HF]^-$	15	486 (14.5), 197 (48.0)	12.31
17α-Hydroxyprogesterone	196[b]	611 $[M\text{-}PFB]^-$	45	772 (15.4), 611 (55.8), 521 (31.1)	13.15
Deoxycorticosterone	611	611 $[M\text{-}PFB]^-$	50	772 (8.3), 472 (18.4), 196 (30.4)	13.55
Corticosterone	699	699 $[M\text{-}PFB]^-$	850[c]	860 (10.2), 196 (68.0)	16.35
Hydrocortisone	196[b]	787 $[M\text{-}PFB]^-$	40	787 (98.8), 698 (41.3)	17.34

[a] LOD, limit of detection.
[b] The ion m/z is a reagent specific ion peak.
[c] The higher limit of detection for corticosterone may be due to the amount of unlabelled steroids in internal standard (i.e. 3.0%).

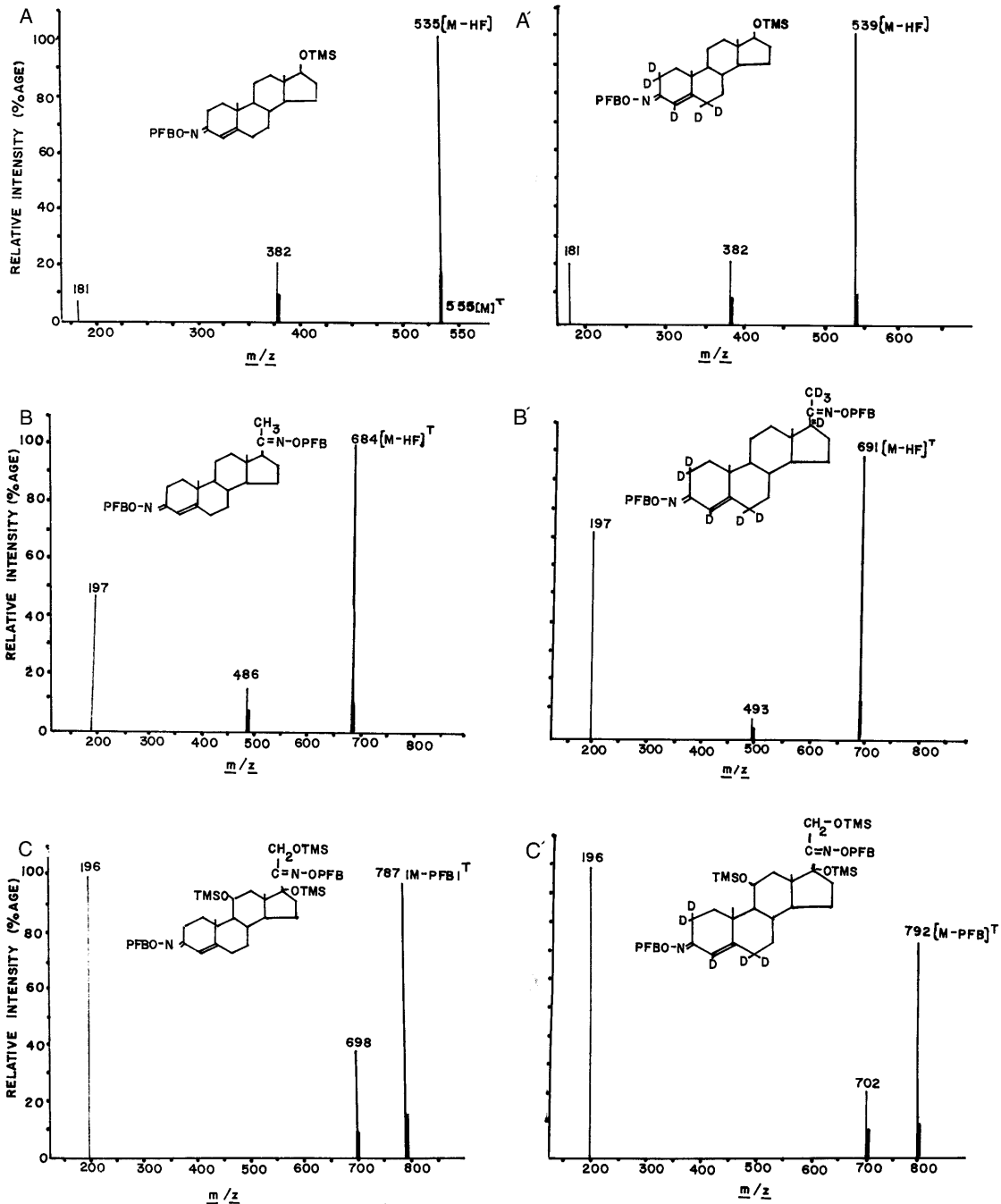

Fig. 1. The NICI mass spectrum of: A & A′; testosterone and [$^2H_5$]-testosterone, B & B′; progesterone and [$^2H_7$]-progesterone, C & C′; hydrocortisone and [$^2H_5$]-hydrocortisone, respectively.

Table 2
Isotopomeric composition of deuteriated internal standards

Compound	Ion monitored	Isotopomeric composition
Testosterone	$[M\text{-}HF]^-$	$^2H_5$ (40.0%), $^2H_4$ (38.4%), $^2H_3$ (17.0%), $^2H_2$ (3.8%), $^2H_1$ (0.46%), $^2H_0$ (0.03%)
4-Androstene-3,17-dione	$[M\text{-}HF]^-$	$^2H_7$ (48.5%), $^2H_6$ (39.0%), $^2H_5$ (12.5%), $^2H_4$-$^2H_0$ (0%)
Progesterone	$[M\text{-}HF]^-$	$^2H_7$ (21.1%), $^2H_6$ (34.9%), $^2H_5$ (28.0%), $^2H_4$ (12.8%), $^2H_3$ (3.1%), $^2H_2$-$^2H_0$ (0%)
17α-Hydroxyprogesterone	$[M\text{-}PFBO]^-$	$^2H_8$ (42.0%), $^2H_7$ (36.2%), $^2H_6$ (16.8%), $^2H_5$ (6.0%), $^2H_4$-$^2H_0$ (0%)
Deoxycorticosterone	$[M\text{-}PFBO]^-$	$^2H_6$ (49.0%), $^2H_5$ (38.6%), $^2H_4$ (12.0%), $^2H_3$ (0.5%), $^2H_2$-$^2H_0$ (0%)
Corticosterone	$[M\text{-}PFBO]^-$	$^2H_6$ (64.7%), $^2H_5$ (22.8%), $^2H_4$ (3.8%), $^2H_3$ (0.8%), $^2H_2$ (1.5%), $^2H_1$ (3.0%), $^2H_0$ (3.0%)
Hydrocortisone	$[M\text{-}PFBO]^-$	$^2H_5$ (53.0%), $^2H_4$ (22.4%), $^2H_3$ (20.0%), $^2H_2$ (3.8%), $^2H_1$ (0.5%), $^2H_0$ (0.0%)

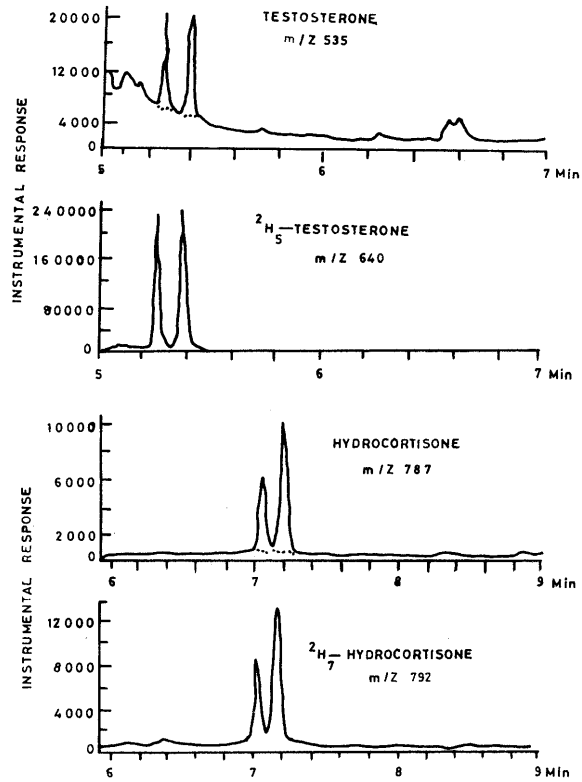

Fig. 2. GC-NICIMS-SIM traces of endogenous hydrocortisone and testosterone in bovine aqueous humour (4 ml) following the addition of corresponding deuteriated internal standards (10 ng), extraction and formation of the PFBO-TMS derivatives.

Table 3
Mean concentrations (ng ml^{-1}) of steroids in bovine aqueous ($n = 17$) and vitreous humour ($n = 19$)[a]

Steroid	Aqueous humour		Vitreous humour	
	n'	Mean (±S.E.M.)	n'	Mean (±S.E.M.)
Testosterone	4	0.14 (0.04)	0	BLD
4-Androstene-3,17-dione	8	0.15 (0.07)	19	0.11 (0.02)
Progesterone	15	0.06 (0.01)	18	0.09 (0.01)
17α-Hydroxyprogesterone	0	BLD	6	5.55 (3.12)
Hydrocortisone	17	2.39 (0.54)	19	1.78 (0.25)

[a] n, no. of samples; BLD, below limit of detection; and n', no. of samples in which the steroid was detected.

2.3. Preparation of deuteriated internal standards

2.3.1. Synthesis of $[^2H_5]$-testosterone, $[^2H_7]$-progesterone and $[^2H_7]$-4-androstene-3,17-dione

The deuterium labeled isotopomers of the steroids was prepared by dissolving the corresponding standard steroids (200 mg) in the solution of potassium *tert* butoxide (2 ml; 0.05 M in $C_2H_5O^2H$; w/v) and heated at 60°C for 4 h with occasional shaking. However, the similar solution of deoxycorticosterone, corticosterone or 17α-hydroxyprogesterone were kept in dark for 5 h, at room temperature. Then ^2HCl (0.2 ml; 35% w/v) was added and the mixture was shaken for a few minutes. Ethanol was removed under a stream of

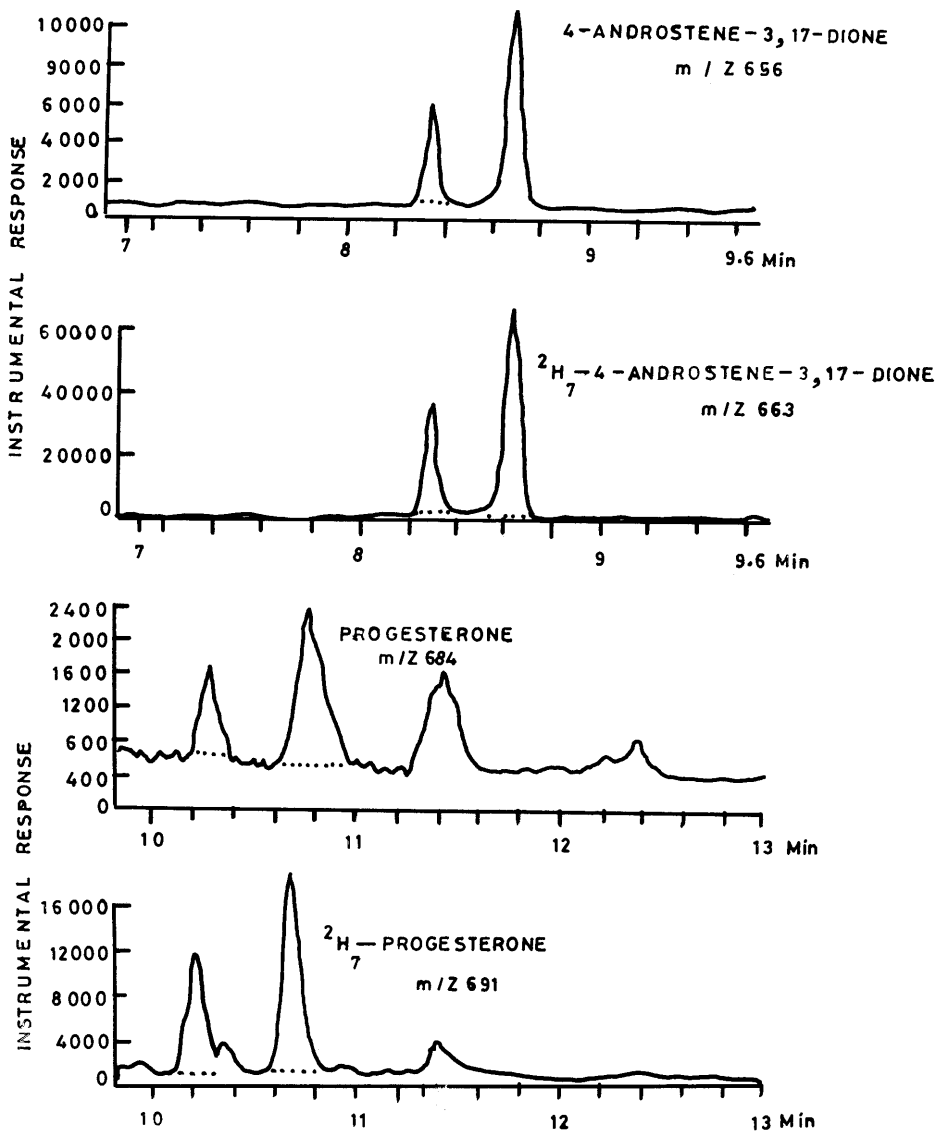

Fig. 3. GC-NICIMS-SIM traces of endogenous 4-androstene-3,17-dione and progesterone in bovine vitreous humour (4 ml) following the addition of corresponding deuteriated internal standards (10 ng), extraction and formation of the PFBO-TMS derivatives.

nitrogen and ^2H_2O (5 ml) was added. The deuteriated product was extracted into ethyl acetate (2 × 5 ml) and combined organic layers were dried over anhydrous sodium sulphate. The solvent was removed by evaporation under reduced pressure. The residue was redissolved in a small volume of ethyl acetate and hexane was added to effect recrystallisation.

The PFBO-TMS derivatives of the labelled steroids were prepared and injected into the GC-MS under NICI mode for characterization. These were used as internal standard without further purification. [$^2H_5$]-Hydrocortisone was synthesized by the method described elsewhere [18].

2.3.2. Preparation of PFBO-TMS derivatives of standard steroids

The steroid (50 μg) was treated with PFBO HCl (100 mg ml^{-1}; in dry pyridine; 30 μl) at 60°C for 15 min, TMS IM (40 μl) was then added and heated at 60°C for further 6 h. Mixture was then kept in dark at room temperature for 48 h to complete the reaction. The reaction mixture was then diluted with ethyl acetate (200 μl), shaken for 1 min and further diluted with hexane to 1 ml. The mixture was centrifuged (1500 × *g* for 3 min) to remove the precipitate, the supernatant was passed through a short column of Sephadex LH-20 (ca ≈3 cm in a Pasteur pipette) and the column was washed with hexane (1 ml). The volume of the eluent was reduced to 0.5 ml under a stream of nitrogen and 2 μl was injected into the GC-MS.

2.4. Biological samples

Eyes from freshly killed cattle were obtained from a local abattoir. Aqueous humour (ca ≈1 ml) and vitreous humour (ca ≈4–5 ml) were collected from the eyes with-in 1 h of death of the animal. The samples were stored in vials with screw caps at −20°C, until required for analysis.

2.5. Extraction and derivatisation of endogenous steroids

2.5.1. Aqueous humour and vitreous humour

Accurately measured volume (5 ml) of aqueous humour or vitreous humour was transferred to a test tube with a screw cap. An aliquot of a standard solution (10 μl; 1 ng μl^{-1} of each in acetonitrile) of deuteriated steroids: testosterone, 4-androstene-3,17-dione, progesterone, 17α-hydroxyprogesterone, corticosterone, deoxycorticosterone and hydrocortisone were added. The sample was then agitated for 5 min to disperse the standards.

Aqueous layer was extracted with ethyl acetate (2 × 5 ml) and the combined organic layer was dried by passing through a short column of anhydrous sodium sulphate (ca ≈3 cm in a Pasteur pipette). Ethyl acetate was then evaporated under the stream of nitrogen and the residue was converted to PFBO-TMS derivatives. The final volume of the derivatized sample was reduced to 2 μl and whole sample was injected into the GC-MS.

2.6. Calibration curves

These were constructed by adding the fixed amount of the solution of deuteriated standard steroid (5 ng, i.e. 5 μl; 1 ng μl^{-1} in acetonitrile) and varying quantities of the corresponding unlabelled standard steroid over the concentration range of 0.5–10 ng (0.25 ng μl^{-1} in acetonitrile) to water (1 ml). The samples were then extracted and derivatised as described above for biological samples. The instrumental response was found to be linear over the range of 0.5–10 ng. Where syn- and anti-isomers were present the area of both peaks were added to construct the calibration curve.

3. Results and discussion

3.1. Preparation of PFBO-TMS derivatives of steroids

Despite of using the longer reaction time and various temperature conditions it was not possible to improve the yield of the PFBO-TMS derivatives of steroids as reported earlier [17]. The strong electron capturing properties of the PFBO

moiety compensated the poor yield of these derivatives molecule, under NICI conditions [17].

The base peak and other significant ions of the PFBO-TMS derivatives of the steroids under NICI conditions are shown in Table 1. The mass spectra of PFBO-TMS derivative of hydrocortisone and 17α-hydroxyprogesterone gave the base peak at m/z 196, a reagent (PFBO) specific ion peak. The other significant ions for PFBO-TMS derivative of hydrocortisone were at m/z 787 $[M\text{-}PFBO]^-$ and for the PFBO-TMS 17α-hydroxyprogesterone were at m/z 772 $[M\text{-}HF]^-$ and at m/z 611 $[M\text{-}PFB]^-$. The base peaks for PFBO-TMS derivatives of corticosterone and deoxycorticosterone were at m/z 699 $[M\text{-}PFBO]^-$ and at m/z 611 $[M\text{-}PFB]^-$, respectively. However, the base peak for progesterone, testosterone and 4-androstene-3,17-dione were due to the loss of hydrogen fluoride $[HF]^-$ from the molecular ions. Typical mass spectra of the PFBO-TMS derivatives of testosterone, progesterone and hydrocortisone are shown in Fig. 1.

The PFBO-TMS derivatives of these steroids under NICI conditions show the diagnostic ions in high abundance which are suitable for the analysis of low concentrations of these steroids in biological samples. The ion used for selected ion monitoring (SIM), GC retention times and the limits of detection for the PFBO-TMS derivatives of these compounds are summarized in Table 1.

3.2. Mass spectra of deuteriated standards

Deuteriated isotopomers of testosterone, 4-androstene-3,17-dione, progesterone, 17α-hydroxyprogesterone, corticosterone and deoxycorticosterone were yellow crystalline solids but, under similar conditions, hydrocortisone failed to form deuteriated isotopomers which may be due to its decomposition. Then, it was synthesized by the method described elsewhere [17].

The isotopomeric composition of PFBO-TMS derivative of labeled steroids determined by GC-NICIMS is shown in Table 2. The typical GC-NICIMS spectra of labelled and unlabelled testosterone, progesterone and hydrocortisone are shown in Fig. 1. The composition of $[^2H_0]$ isotopomer was $< 0.1\%$ except in the labeled corticosterone where the amount of $[^2H_0]$ was about 3.0% but this was within acceptable limits. Thus, the labeled steroids prepared were suitable for use as internal standards.

3.3. Quantification of corticosteroids, androgens and progestogens in bovine eye aqueous humour

3.3.1. Aqueous humour

Bovine aqueous humour ($n = 17$) was analyzed simultaneously for the presence of testosterone, 4-androstene-3,17-dione, progesterone, 17α-hydroxyprogesterone, corticosterone, 11-deoxycorticosterone and hydrocortisone. Before the analysis of biological samples, a reagent blank sample (containing only deuterated standard of the labeled steroid; 5 ng of each) was checked in order to eliminate the possibility of any contamination and a standard 1:1 mixture of labeled and unlabelled standard steroids (containing 5 ng of labeled and unlabelled of each steroid) was analyzed at the end of each batch of biological samples.

Hydrocortisone was determined in all samples of bovine aqueous humour with a mean ($\pm$ S.E.M.) concentration of 2.39 ± 0.54 ng ml^{-1} (range of concentration of hydrocortisone in bovine aqueous humour was 0.86–8.83 ng ml^{-1}); progesterone was detected in 15 samples of bovine aqueous humour with a mean ($\pm$ S.E.M.) concentration of 0.06 ± 0.006 ng ml^{-1}, with a range of 0.02–0.10 ng ml^{-1}; 4-androstene-3,17-dione was detected in only eight samples of bovine aqueous humour with a mean ($\pm$ S.E.M.) concentration of 0.15 ± 0.07 ng ml^{-1}, with a range of 0.01–0.56 ng ml^{-1} and, in the rest of the samples, the amounts of steroids were below the limit of detection.

Despite a very low limit of detection (20 pg) for testosterone, it was detected in four samples of bovine aqueous humour with a mean ($\pm$ S.E.M.) concentration of 0.14 ± 0.04 ng ml^{-1} with a range of 0.07–0.18 ng ml^{-1}.

The concentrations of corticosterone, deoxycorticosterone and 17α-hydroxyprogesterone were below the limits of detection in all the samples analysed. Table 3 shows the concentrations of steroids in bovine aqueous humour and Fig. 2 shows the SIM traces of derivatized hydrocortisone and testosterone in bovine aqueous humour.

3.3.2. Vitreous humour

Analysis of bovine vitreous humour ($n = 19$) showed the presence of hydrocortisone in all samples with a mean ($\pm$S.E.M.) concentration of 1.78 ($\pm$0.25 ng ml^{-1}) and with a range of 0.07–4.35 ng ml^{-1}; 4-androstene-3,17-dione was detected in all 19 samples of bovine vitreous humour with a mean concentration ($\pm$S.E.M.) of 0.11 ($\pm$0.02 ng ml^{-1}), with a range of 0.02–0.38 ng ml^{-1}; progesterone was detected in 18 samples of bovine vitreous humour with a mean ($\pm$S.E.M.) concentration of 0.09 ($\pm$0.01 ng ml^{-1}) with a range of 0.018–0.25 ng ml^{-1}; 11-deoxycorticosterone was detected in 12 samples of bovine vitreous humour with a mean ($\pm$S.E.M.) concentration of 29.27 ($\pm$6.42 ng ml^{-1}) with a range of 3.58–63.76 ng ml^{-1} and 17α-hydroxyprogesterone was detected in six samples of bovine vitreous humour with a mean ($\pm$S.E.M.) concentration of 5.55 ($\pm$3.12 ng ml^{-1}) with a range of 0.31–19.89 ng ml^{-1}.

The concentrations of testosterone and corticosterone were below the limit of detection (20 and 850 pg, respectively) of this method. The concentrations of steroids determined in vitreous humour are shown in Table 3 and SIM traces of the derivatized 4-androstene-3,17-dione and progesterone from bovine vitreous humour are shown in Fig. 3.

These results show that the concentrations of hydrocortisone, 4-androstene-3,17-dione and progesterone in aqueous humour are similar to those in vitreous humour.

The concentrations of corticosteroids determined in rabbit's aqueous humour by the tetrazolium blue assay were very high (2000–4000 ng ml^{-1}) [12]. This method is nonspecific and it is possible that the reagent may reacts with other materials present in the aqueous humour. The concentration of hydrocortisone determined by RIA [16] and GC-NICIMS [17] in human aqueous humour were 32.0 and 4.9 ng ml^{-1}, respectively. RIA methods are very sensitive but are limited by the non-specificity of antibodies used. The concentration of corticosterone determined in rabbit aqueous humour by RIA was 7.76 ng ml^{-1} [15], while the present work showed that it was below the limit of detection (40 pg) in bovine aqueous humour.

The concentration of testosterone in rabbit aqueous humour determined by RIA was found to be 0.82 and 0.75 ng ml^{-1} in male and female rabbits, respectively [19]. In the current studies, despite of the high sensitivity (detection limit 20 pg) of the GC-NICIMS method, testosterone was detected in only four out of 17 samples of bovine aqueous humour.

In present study, the number of endogenous steroids have been quantified both in bovine aqueous and vitreous humour. The presence of endogenous steroids in aqueous and vitreous humour may play an important role in the homeostasis of aqueous humour, e.g. maintenance of glucose level, ion transport and IOP. The determination of the endogenous steroids and their metabolites in aqueous humour, vitreous humour and other parts of the eye from glaucomatous and non-glaucomatous may help in understanding the role of steroids in maintaining IOP.

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