The metabolism of fluorometholone by bovine cornea*

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Abstract: Isolated bovine cornea was found to reduce the 20 ketone group in fluorometholone (FML) to produce a mixture of 20α- and 20β-dihydrofluorometholone (DHFML). Incubation of FML with bovine cornea at pH 7.4 in phosphate buffer produced an increase in the amount of reduction products up to 8 h; after this time the rate of reduction decreased. The reduced products were quantified by GC–MS against [^{2}H$_3$] 20α-DHFML. Variable amounts of 20β-DHFML were detected in six samples of human aqueous humour following topical application of 50 μg of FML to the eye. The rapid metabolism of the side chain of FML may account for the unexpected low concentrations of FML which are found in human aqueous humour following its application to the surface of the eye.

Keywords: Fluorometholone metabolism; corneal ketoreductase; GC–MS.

Introduction

Topical administration of corticosteroids is widely used in the treatment of ocular inflammation produced both by infection and as a consequence of surgery. Recently the concentrations of synthetic corticosteroids in human aqueous humour have been measured following topical application of a number of ocular corticosteroid formulations [1–5]. Our results indicated that the amounts of different corticosteroids penetrating into the human eye were much lower than the amounts determined earlier in a rabbit model [6, 7]; in the rabbit studies the amount of radiolabelled steroid penetrating into the aqueous humour was determined. Since chromatography was not carried out, the degree of metabolism of the steroids could not be assessed. However, in one study thin layer chromatography was carried out on the radiolabelled compounds accumulating in rabbit aqueous humour following topical application of the steroids [8]. In this case unidentified metabolites of fluorometholone, dexamethasone and prednisolone acetate were observed when TLC analysis of radiolabelled metabolites was carried out; fluorometholone was found to be extensively metabolized. Our recent studies of the penetration of corticosteroids into the human eye were carried out using gas chromatography–mass spectrometry with stable isotope dilution, thus the analysis was much more specific than simple use of a radiolabel. In these studies it was found that the lowest degree of penetration into the eye was achieved by fluorometholone (FML) [5]. FML is one of the more lipophilic steroids used in eye drop preparations and would be expected to penetrate the eye effectively. The low degree of penetration observed conforms with the findings that FML does not have a marked effect on intra-ocular pressure [9]. The side chain of FML differs from that of the other corticosteroids used in eye drops which have a dihydroxyacetone side chain. Thus it would seem possible that the relatively low concentrations of FML found in human aqueous humour are a result of rapid metabolism of the FML side chain on passing through the cornea. In this paper the metabolism of FML by bovine cornea is reported and also some preliminary data for the analysis of FML metabolites in human aqueous humour.

Materials and Methods

Chemicals

Chemicals were obtained from the following sources: pentafluorohydroxylamine hydro-

chloride (PFBO.HCl), sodium borohydride, silica gel 230–400 mesh (Aldrich, Gillingham, UK); Sephadex LH20 (Sigma, Poole, UK); trimethylsilyl–imidazole (TMS-IM) (Fluka, Gillingham, UK); octadecyltrimethoxysilane (Fluorochem, Glossop, UK). HPLC grade ethyl acetate, hexane and methanol (Rathburn, Walkerburn, UK). Fluorometholone was a gift from Allergan Pharmaceuticals (High Wycombe, UK).

Preparation of standards

20α-dihydrofluorometholone (20α-DHFML) was prepared by dissolving FML (100 mg) in methanol (500 ml), the mixture was kept at 0°C in an ice bath and then sodium borohydride (25 mg), was added. The mixture was stirred at 0°C for 90 min. Glacial acetic acid (100 μl) was added to the reaction mixture and the methanol was removed under reduced pressure. The product was extracted from the residue with ethyl acetate and crystallized to yield white crystals m.p. 246°C (yield 70%). The IR (KBr disc) differed from that of FML in that the band due to unconjugated CO stretching at 1710 cm^{-1} was absent. NMR was run on a Bruker 400 MHz NMR in [^{2}H$_5$] pyridine and the following signals could be assigned from the COSY spectrum: δ 1.06 (d, J = 6.3 Hz, 3H, H-22); δ 1.37 (d, J = 6.7 Hz, 3H, H-21); δ 1.46 (s, 3H, H-19); δ 1.79 (s, 3H, H-18); δ 1.25–1.4 (m, 2H, H15α,β); δ 1.7–1.9 (m, 4H, H-7 α,β, H-8, H-14); δ 2.57–2.8 (m, 5H, H-6, H12 α,β, H-16 α,β); δ 4.28 (quintet, J = 6 Hz, 1H, H-20); δ 4.67 (d, J = 10.6 Hz, 1H, H-11); δ 4.71 (s, 1H, OH-17); δ 5.48 (d, J = 6 Hz, 1H, OH-20); δ 6.37 (s, 1H, OH-11); δ 6.55 (dd, J = 9.5 Hz, 1.8 Hz, 1H, H-1); δ 6.72 (t, J = 1.7 Hz, 1H, H-4); δ 7.58 (d, J = 9.5 Hz, 1H, H-2) ppm.

20β-dihydrofluorometholone (20β-DHFML) was prepared by dissolving FML (100 mg) in THF (150 ml). The mixture was cooled in ice and then NaBH$_4$ (25 mg) was added and stirring was continued for a further 2 h. Acetic acid (100 μl) was added and the THF was removed by rotary evaporation. The residue was taken up in chloroform. A 1:1 mixture of the 20α- and 20β-DHFML was generated by this procedure and the two products were separated by chromatography on octadecylsilica gel (prepared by coating flash column silica gel using octadecyltrimethoxy silane) in methanol–water (70:30). Yield of 20β-isomer after separation 32%. The 20β-

DHFML isolated lacked an unconjugated CO stretching band in its IR spectrum and the following bands could be assigned in its 400 MHz COSY NMR spectrum run in [^{2}H$_5$] pyridine: δ 1.05 (d, J = 6.3 Hz, 3H, H-22); δ 1.29 (s, 3H, H-19); δ 1.48 (d, J = 6.2 Hz, 3H, H-21); δ 1.79 (s, 3H, H-18); δ 2.07 (d, J = 14.6 Hz, 1H, H-12β); δ 2.24 (m, 1H, H-16β); δ 2.44 (m, 1H, H-16α); δ 2.81 (d, J = 14.6 Hz, 1H, H-12α); δ 4.18 (quartet, J = 6.2 Hz, 1H, H-20); δ 4.72 (d, J = 10.2 Hz, 1H, H-11); δ 4.88 (s, 1H, OH-17); δ 5.4 (broad s, 1H, OH-20); δ 6.37 (s, 1H, OH-11); δ 6.56 (dd, J = 10.2 Hz, 1.8 Hz, 1H, H-1); δ 6.69 (t, J = 1.7 Hz, 1H, H-4); δ 7.56 (d, J = 10.2 Hz, 1H, H-2) ppm.

[^{2}H$_3$] 20α-DHFML

FML was deuteriated at C-21 by heating 50 mg of FML in a solution containing 60% of deuteriated glacial acetic acid in D$_2$O (5 ml) at 120°C in a sealed tube for 12 h. The glacial acetic acid solution was then cooled, diluted with 10 ml of water and the deuteriated FML was extracted into ethyl acetate, dried and the solvent was removed under vacuum. The [^{2}H$_3$] FML formed was then reduced with sodium borohydride as described for the preparation of 20α-DHFML. The isotope composition determined from GC–MS was as follows: [^{2}H$_3$] 80.9%; [^{2}H$_2$] 15.6%; [^{2}H$_1$] 3.5%.

Incubation of FML with bovine cornea

Bovine eyes were obtained from freshly killed cattle and the cornea was removed with a scalpel.

FML (50 μg) was suspended in 1 ml of potassium phosphate buffer (pH 7.4, 1 M) and the suspension was incubated with a weighed cornea. Initially incubations were carried out at pH 4.9 in sodium acetate buffer and pH 7.0, 7.4 and 8.0 in potassium phosphate buffers for 6 h in order to determine the optimal pH for metabolism. Following incubation 250 ng of deuteriated 20α-DHFML internal standard was added, the corneas were homogenized in a ground glass homogenizer and the homogenate was extracted with ethyl acetate (2 × 2 ml), the extract was dried with anhydrous Na$_2$SO$_4$. Control incubations of FML for 6 h in potassium phosphate buffer in the absence of cornea were also carried out. The incubation of FML with cornea was carried out for the following times: 0, 5, 15, 30, 120, 240, 360, 480 and 1440

min and following incubation the samples were processed as described above.

Analysis of DHFML in human aqueous humour

FML (50 μg in 50 μl) in a commercial eye drop preparation (FML, Allergan) was applied to the eyes of patient volunteers undergoing cataract surgery at various time intervals before surgery. During surgery samples of aqueous humour were collected as described previously [1] and were stored frozen until analysis. They were then diluted with 1 ml of water, 10 ng of deuteriated 20α-DHFML was added, the solution was extracted with ethyl acetate (2 × 1 ml) and the extract was dried with anhydrous Na_2SO_4.

Derivatization of samples

Ethyl acetate was removed from the extracts under a stream of nitrogen and pentafluoro-benzylhydroxylamine (PFBO) hydrochloride solution (100 mg ml^{-1} in dry pyridine, 30 μl) was added to the residue. The solution was heated (30 min at 60°C) and then trimethylsilyl imidazole (30 μl) was added and the mixture was heated for a further 2 h at 60°C. The mixture was diluted with ethyl acetate (100 μl) followed by hexane (1 ml) and was then passed through ca 3 cm of Sephadex LH 20 in a Pasteur pipette. In the case of the aqueous humour extracts the sample was reduced to ca 5 μl and the whole sample was injected into the GC–MS. In the case of extracts from incubations injections were made from a final volume of 100 μl.

Instrumentation

GC–MS analysis was carried out in the negative ion chemical ionization (NICI) mode using a HP 5988 GC–MS system. Methane was used as the reagent gas with a source pressure of ca 1 Torr. The source temperature was 140°C and the electron energy 200 eV. The GC was fitted with a Restek Rtx-1 column (15 m × 0.25 mm i.d. × 0.25 μm film, Belmont Instruments, Glasgow), helium was used as the carrier gas with a head pressure of 0.25 bar. The injection was via a Grob splitless injection system, the injector temperature was 250°C and the interface temperature was 280°C. The GC was programmed as follows: 190°C (1 min) then 20°C min^{-1} to 300°C (10 min).

Calibration

Calibration curves for varying amounts of 20α- and 20β-DHFML against a fixed amount of [$^2H_3$] 20α-DHFML were constructed over the ranges 0.5–10 ng and 50–1000 ng and were found to be linear over these ranges.

Results and Discussion

Assignment of the structures of the synthetic standards

Figure 1 shows the structures for FML, 20α-DHFML (20S-DHFML) and 20β-DHFML (20R-DHFML). The use of NOESY to fully
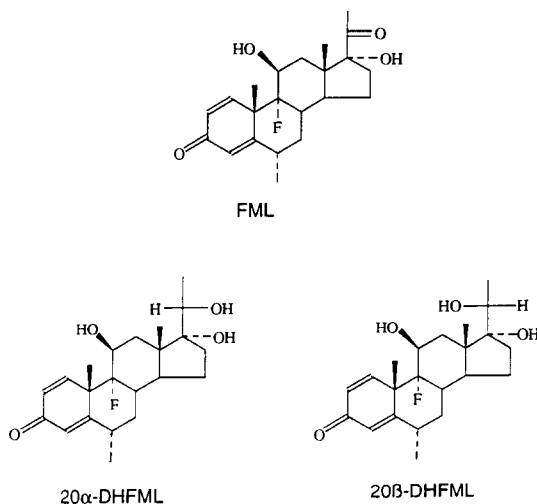

Figure 1
The structures of FML and 20α- and 20β-DHFML.

elucidate the structures of the two 20-DHFML standards did not result in a definitive answer since the protons of the methyl at 19 exhibited a NOE with the 20 proton in both isomers. Tentative assignments of the 20-DHFML standards have been made as described below. Definitive assignments will probably require ORD and CD measurements on the two products in comparison with such data for related steroids of defined stereochemistry.

NMR spectra were determined in pyridine so that intramolecular hydrogen bonding would be favoured where it could occur. This would be favoured for 20β-DHFML where the 21 methyl group would be held away from the 19 methyl group if intramolecular hydrogen bonding occurred. There are few recent examples of the direct determination of the stereochemistry of the products from reduction of 20-ketosteroids and most assignments rely on early precedents established in the literature; it has been reported that reduction with NaBH₄ tends to favour the 20β derivative [10]. Intramolecular H-bonding in 20α-DHFML would be less favourable since it would entail the 21 methyl group being brought into close proximity to the 19 methyl group which is sterically unfavourable. On the basis of the NMR data it would seem likely that the single product resulting from the reduction of FML in methanol with NaBH₄ is 20α-DHFML and that the product isolated from the 50:50 mixture formed when reduction was carried out in THF is 20β-DHFML. In the NMR spectrum of the product presumed to be 20β-DHFML the signal due to the methylene protons in the steroid nucleus are spread over a wide range of field shift. This would occur if intramolecular hydrogen bonding had caused the hydroxyl groups at the 17 and 20 positions to be on the α face of the molecule thus producing greater asymmetry between the α and β faces of the molecule. Further support for product isolated from the reduction in THF being 20β-DHFML is given by the signal due to the 20 OH proton which is very broad and does not couple to the proton on the 20 carbon which consequently appears as a quartet. This would occur where the environment of the proton was changeable due to it being bonded in varying degrees to the oxygen of the 17 OH group. The product from the reduction in methanol gave a sharp signal for the 20 OH proton which appeared as a doublet and which was coupled to the proton on the 20 carbon causing its signal to appear as

a quintet. In addition the signals due to the methylene protons in the steroid nucleus were confined to a narrow range of field shift.

Incubation of FML with bovine cornea

Figure 2 shows the mass spectrum of the PFBO–TMS derivative of 20α-DHFML; the mass spectrum of the PFBO–TMS derivative of 20β-DHFML was very similar. The base peak in the mass spectra results from the loss of PFB from the molecular ion of the derivative. The base peaks (*m/z* 608) in the mass spectra were used to monitor the formation of the two metabolites using GC–MS with SIM.

The optimum pH for the formation of 20α- and 20β-DHFML was established as pH 7.4. Figure 3 shows a SIM trace for a 6 h incubation of bovine cornea with 50 μg of FML showing
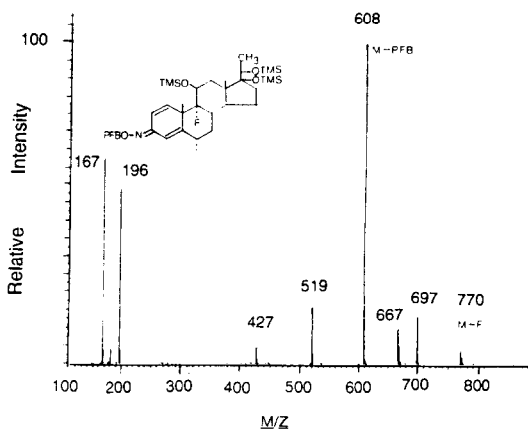
Figure 2
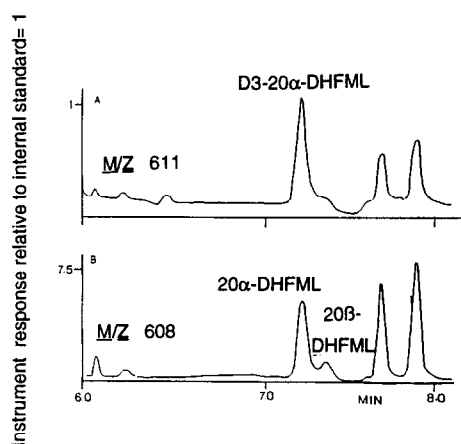The NICI mass spectrum of 20α-DHFML PFBO-TMS derivative.

Figure 3
SIM trace of PFBO-TMS derivatives showing the formation of 20α- and 20β-DHFML after a 6 h incubation of FML with bovine cornea (B). A 250 ng mass of [²H₃] 20α-DHFML was added prior to extraction and derivatization (A).

peaks for 20α- and 20β-DHFML in comparison with 250 ng of [^{2}H$_3$] 20α-DHFML added as an internal standard. The rate of formation of 20α- and 20β-DHFML with time is summarized in Fig. 4. The metabolites reached a plateau at about 8 h when approximately 6% of the substrate had been reduced; presumably further reaction was inhibited by the DHFML metabolites which would partition favourably into the lipophilic environment of the cornea or alternatively an enzymatic co-factor may have become depleted. Figure 5 shows a SIM trace for a 6 h incubation of bovine cornea without addition of FML showing an absence of peaks for DHFML and the presence of two additional peaks which are naturally present in bovine cornea and not additional metabolites of FML. In a previous study the stereospecific formation of 20α-DHFML by rabbit cornea

Figure 4
The rate of formation of 20α- and 20β-DHFML with time when FML was incubated with bovine cornea (*n* = 3 for each time point).

Figure 5
SIM trace showing a derivatized extract from bovine cornea incubated for 6 h in the absence of FML. A 250 ng mass of [^{2}H$_3$] 20α-DHFML was added prior to extraction and derivatization (A).

was observed when FML was topically applied to rabbit eye [11].

Metabolism of FML in the human eye

We have obtained some preliminary evidence for the formation of 20β-DHFML in the human eye. Five samples of human aqueous humour (Table 1) were found to contain DHFML after topical application of FML but in nine other samples the metabolite could not be detected. Unlike the *in vitro* metabolism of FML by bovine cornea the metabolism *in vivo* in the human eye appeared to be stereospecific, producing 20β-DHFML. The inconsistent detection of DHFML in human aqueous humour may be due to metabolism of FML via more than one route. The reduction of the 20-keto group of progesterone by human cornea has previously been reported [12, 13]. However, other routes of metabolism for oral doses of FML have been observed including those leading to the formation of 6,7-dehydro FML and 6β-hydroxy FML [14].
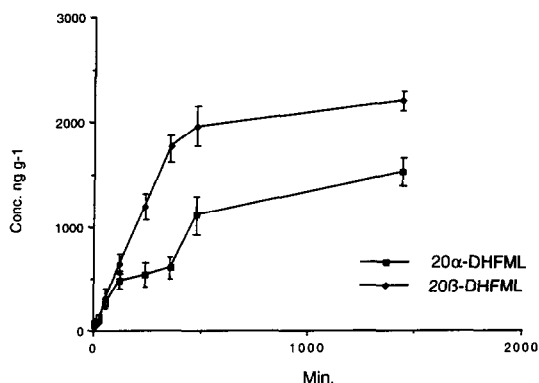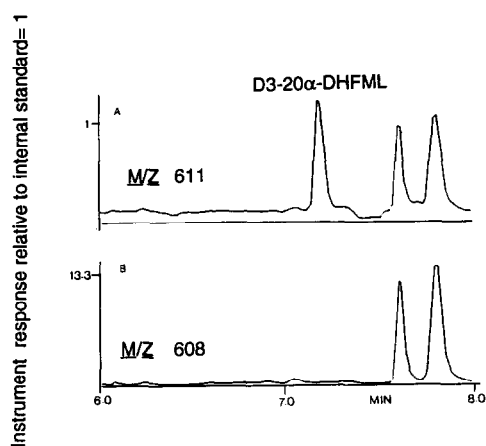
Table 1
Concentration of 20β-DHFML in human aqueous humour samples at various times after topical administration of 50 µg of FML in a commercial eye drop preparation

Time (min)	Conc. of 20β-DHFML (ng ml^{-1})
65	39.8
115	146.7
195	20.0
610	13.2
640	10.2

Of the steroids studied in earlier work FML was anomalous in its penetration into the eye [5]. The concentrations of FML found in the aqueous humour following topical application to the human eye were much lower than those that would have been predicted on the basis of its lipophilicity and the concentration in the drop. In our earlier work the standard deviations for the aqueous humour concentrations of the other topically applied corticosteroids studied were quite large [1–4]. It is possible that such variations might be accounted for by variations in rates of corneal steroid metabolism between individuals. Variations in metabolism by the cornea may be an important factor governing whether or not individuals respond to steroids with an excessive rise in intra-ocular pressure and their susceptibility to steroid induced glaucoma. The biological activity of steroid metabolites would also be of

importance in determining the overall action of a given steroid. Fluorometholone has only a low anti-inflammatory effect in the eye but its 17-acetate is much more potent [15]; this is presumably because the acetate group hinders the reduction of the 20-keto group. Esterases in the cornea might eventually remove the acetate group leaving the 20-keto group vulnerable to reduction but such a two-stage deactivation would be much slower.

A knowledge of ocular metabolism is essential in order to make a full assessment of the overall effectiveness of any drug used in ophthalmological practice. Surprisingly there is very little in the literature on metabolism of drugs by the eye. In the course of our studies of drug penetration into the eye we have observed metabolites due to the action of esterase [3], reductase [16] and amine oxidase enzymes [17]. A knowledge of drug metabolism in the human eye would also facilitate the design of prodrugs which could take advantage of metabolic activation and improve the delivery of an active form of a drug into the eye.

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