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The generation of fluorometholone nanocrystal eye drops, their metabolization to dihydrofluorometholone and penetration into rabbit eyes

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ABSTRACT

Fluorometholone is a widely used anti-inflammatory ophthalmic formulation, which elicits a lower ocular hypertensive response than other glucocorticoid medications. This serves to mitigate against the risk of steroid-induced glaucoma. Based on the hypothesis that an improved corneal permeability can increase the bioavailability of a drug, we sought to obtain fluorometholone in suspension with a small particle size. Accordingly, we describe the formulation of fluorometholone nanocrystal eye drops, which have a mean particle size of 201.2 ± 14.1 nm (standard deviation (s.d.)) when measured by dynamic light scattering. Scanning electron microscopy further indicates that fluorometholone nanocrystals are predominantly rectangular in shape. Fluorometholone microcrystals, on the other hand, with a mean particle size of 9.24 ± 4.51 μm (s.d.), tend to have a rod-like morphology. Powder x-ray diffraction revealed that fluorometholone microcrystal and nanocrystal formulations have the same crystal structure, with the main diffraction peaks at $2\theta = 10.4$ and 15.3°. The nanocrystal formulation was found to be stable, long-term, when stored at 10 °C for up to 6-months. High pressure liquid chromatography (HPLC) of the aqueous humor of rabbit eyes 15–240 mins after the *in vivo* application of fluorometholone eye drops to the ocular surface revealed that the molecule had been converted to 20α-dihydrofluorometholone (with no evidence of a 20β-dihydrofluorometholone fraction), and that penetration was 2–6 fold higher and longer lasting with the nanocrystal, rather than the microcrystal, formulation. In current study we show how newly generated fluorometholone nanocrystals when administered as eye drops enter the anterior chamber of the eye and become metabolized to dihydrofluorometholone.

1. Introduction

The glucocorticoid, fluorometholone, is widely used as an anti-inflammatory medication for the treatment of keratoconjunctivitis and for the management of post-operative ocular healing. One of the merits of using fluorometholone is that, unlike other glucocorticoid eye drops such as dexamethasone, it is unlikely to lead to steroid-induced ocular hypertension and steroid-induced glaucoma (Jones and Rhee, 2006; Kersey and Broadway, 2006; Mindel et al., 1980). The main actions of glucocorticoids are mediated by the glucocorticoid receptor, which is a ligand-activated transcription factor of the nuclear receptor superfamily (Clark et al., 1996; Patel et al., 2019). As far as pathophysiology is concerned, glucocorticoid-induced ocular hypertension causes defects in the ocular outflow pathway, including physical and mechanical changes in the microstructure of the trabecular meshwork. This leads to increased ocular outflow resistance and elevated intraocular pressure (Clark et al., 1996; Patel et al., 2019).

When fluorometholone is applied to the ocular surface it penetrates into the eye through the cornea, during which time it undergoes a metabolizing reaction to become dihydrofluorometholone (Fig. 1). Specifically, fluorometholone has been found to metabolize into 20α-dihydrofluorometholone in the eyes of rabbits and cows (Akura, 1987; Iqbal et al., 1993; Okuda and Tanaka, 1990), and into 20β-dihydrofluorometholone in the eyes of humans and cows, via a reduction of the

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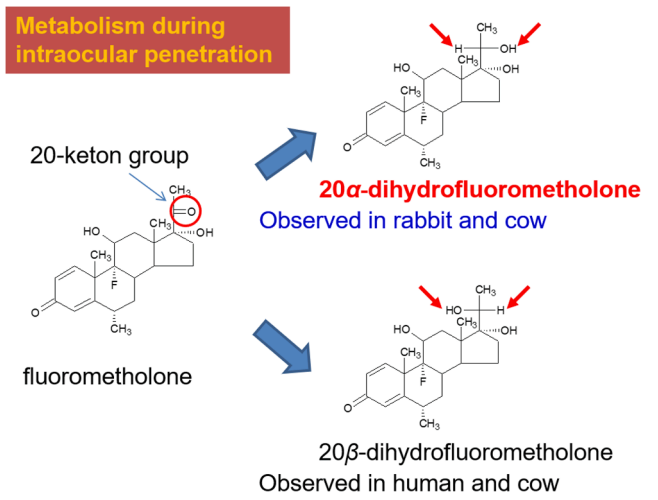

Fig. 1. Schematic showing fluorometholone metabolized to 20*α*- and 20*β*-dihydrofluorometholone.

20-ketone group in fluorometholone to produce 20α- and 20β-dihydrofluorometholone (Iqbal et al., 1993; Kato, 2006). The binding activities of 20α- and 20β-dihydrofluorometholone to the glucocorticoid receptor are, respectively, weak and negligible when compared to the binding activity of fluorometholone (Kato et al., 2006). Thus, the metabolization of fluorometholone to dihydrofluorometholone that arises during its passage through cornea into the aqueous humor is advantageous from the point of view of avoiding the risk of elevated intraocular pressure and steroid-induced glaucoma (Kato et al., 2006; Okuda and Tanaka, 1990), and is consistent with the report that the reduction of the 20-keton group is accompanied by a loss of glucocorticoid activity (Fried and Borman, 1958).

When prescribing eye drops, potential side-effects caused by systemic absorption as a consequence of drug loss through the tear drainage system should be carefully considered. For example, a decrease in endogenous steroid production and a reduction in endogenous plasma cortisol have been attributed to systemic absorption of topically applied dexamethasone eye drops (McGhee, 1992). Moreover, growth suppression in children caused by fluorometholone eye drops has been reported (Wolthers, 2011). To minimize systemic absorption, a means of improving the ocular penetration of topically applied eye drops is required. This can be achieved by applying the drug in a gel or viscous formulation, which increases ocular contact time, or by reducing the size of drug particles that make up the suspension eye drops (Wu et al., 2017; Xiong et al., 2018; Hui and Robinson, 1986; McGhee, 1992). Slow-release from medicated contact lenses is another way to potentially reduce systemic absorption (Hewitt et al., 2020; Morgan et al., 2020). With regards to fluorometholone, it has been shown that the formulation of small particle sizes in the micrometer range increases ocular penetration (Hui and Robinson, 1986; Ueno et al., 1994). Recent research has also described the development of fluorometholone and other glucocorticoid nano eye drops with the aim of increasing the intraocular penetration above that achieved with micrometer sized particles (Yu et al., 2020; Gonzalez-Pizarro et al., 2019a, 2019b, 2018; Noh et al., 2018). However, these studies have not investigated the metabolization of fluorometholone nano eye drops into dihydrofluorometholone, which is important with regards to the bioavailability of the molecule in the cornea and aqueous humor. Here, we report a new methodology for formulating eye drops that contain fluorometholone nanocrystals. The physical properties of these are delineated in terms of particle size, *ζ*-potential, and crystallinity as assessed using scanning electron microscopy (SEM), dynamic light scattering (DLS), and powder x-ray diffraction. The findings are compared to those from parallel studies of fluorometholone microcrystal suspensions. We also report the results of a series of *in situ* experiments to ascertain the concentration of dihydrofluorometholone in the aqueous humor of rabbits after administration of fluorometholone microcrystal versus nanocrystal eye drops.

2. Materials and methods

2.1. Materials

Fluorometholone, *tert*-butanol, lanolin (purified), polyvinylpyrrolidone (PVP), polysorbate 80 (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), hydroxypropyl methyl cellulose (HPMC; Shin-Etsu Chemical Co., Ltd., Tokyo, Japan), and polyoxyethylene (200) polyoxypropylene glycol (70; NOF Corporation, Tokyo, Japan) were used for preparing fluorometholone nanocrystal and microcrystal eye drops. Acetic acid, sodium borohydride, and methanol (HPLC grade; Wako Pure Chemical Industries, Ltd.) were used for synthesizing 20*α*-dihydrofluorometholone and 20*β*-dihydrofluorometholone. Prednisolone and methanol (HPLC grade; Wako Pure Chemical Industries, Ltd.) were used for HPLC measurements. Ketamine (Daiichi Sankyo, Company, Ltd., Tokyo, Japan) and xylazine (Bayer Yakuhin, Ltd., Osaka, Japan) were used for anesthetizing rabbits. Anesthetic and antibacterial eye drops and ointments (oxybuprocaine hydrochloride 0.4% w/w, levofloxacin 1.5% w/w, and ofloxacin 0.3% w/w) were purchased from Santen Pharmaceutical Co., Ltd., Osaka, Japan. All chemicals were used as received.

2.2. Preparation of fluorometholone nanocrystal eye drops

Fluorometholone (120 mg), lanolin (80 mg), and polyoxyethylene (200) polyoxypropylene glycol (70; 80 mg) were dissolved into *tert*-butanol (45 mL) at 60 °C. PVP (80 mg), HPMC (12 mg), and polysorbate 80 (20 μL) were dissolved in purified water (18.2 MΩ, 45 mL: Milli-Q A10; EMD Millipore, Darmstadt, Germany) at 25 °C, and rapidly injected into the *tert*-butanol solution in a flask (45 mL, 1500 rpm, 60 °C), resulting in a milky-white solution (90 mL). This was immediately frozen in liquid nitrogen and freeze dried (FDU-1200, Eyela, Tokyo, Japan) for 24 h to remove the *tert*-butanol solution and water, leaving a dry, white powder, which was re-suspended in purified water (18.2 MΩ, 40 mL) and passed through a 0.8-μm pore size syringe filter (Millipore) to remove aggregates. Finally, the fluorometholone concentration was adjusted to 0.1% w/w (pH 7.0) by dilution with purified water, confirmed by HPLC. The osmolarity of the fluorometholone nanocrystal eye drops was 285.8 mOsm/L, which was ascertained by diffusion/osmosis experiments (ME-6940, Shimadzu Rika, Tokyo, Japan).

2.3. Preparation of fluorometholone microcrystal eye drops

Fluorometholone microcrystal eye drops were prepared using the fluorometholone nanocrystal eye drops as the starting material. First, the nanocrystal eye drops (5 mL) were dissolved into *tert*-butanol (10 mL), mixed with purified water (18.2 MΩ, 100 mL) and stirred for 28 h. During this time, micro-crystallization of fluorometholone was observed. Freezing in liquid nitrogen, followed by a freeze-dry treatment for 24 h to remove *tert*-butanol and water generated a white powder. This was mixed with purified water (5 mL) to form fluorometholone microcrystal eye drops, which contained the same amount of fluorometholone, lanolin, and polymers as the nanocrystal eye drops. As was done for the nanocrystal eye drops, the drug concentration of the microcrystal eye drops was adjusted to 0.1% w/w (pH 7.0), with the osmolarity measured to be 286.0 mOsm/L by diffusion/osmosis experiments (ME-6940, Shimadzu Rika, Tokyo, Japan).

2.4. Scanning electron microscopy (SEM)

The morphology and size of fluorometholone nanocrystals and microcrystals were observed by SEM (JSM-6510LA; JEOL, Tokyo, Japan).

To achieve this, nanocrystal and microcrystal eye drops (50 μL) were subjected to suction filtration with an Isopore™ membrane filter (Type VMTP 0.05-μm pore size, EMD Millipore), after which the membrane was glued onto a SEM stub using conductive tape. Specimens were sputter coated with platinum (JFC-1600, JEOL) and imaged at × 25,000 (nanocrystals) and × 5,000 (microcrystals) magnification.

2.5. Particle size evaluation and zeta (ζ) potential measurements

The mean particle size of nanocrystals in fluorometholone eye drops was measured using a dynamic particle size distribution device (ELSZ-1000; Otsuka Electronics Co. Ltd., Osaka, Japan) in light-scattering mode as per the manufacturer's instructions. Mean nanocrystal particle size (±standard deviation (s.d.)) was calculated using a cumulant analysis method. The mean size of fluorometholone microcrystals was obtained from SEM micrographs, with the long-axis of the particle (n = 191) measured. Average particle size is presented as the mean value ± s.d. The ζ-potentials and polydispersity indices of nanoparticle and microparticle eye drops were measured using the ELSZ-1000 machine in accordance with the manufacturer's instructions.

2.6. Powder x-ray diffraction

To investigate the crystallinity of fluorometholone nanocrystals and microcrystals contained in eye drops, powder x-ray diffraction pattern analysis was carried out using a powder x-ray diffractometer (SmartLab, Rigaku, Tokyo, Japan). Freeze-dried powder samples obtained from nanocrystal and microcrystal eye drops were analyzed alongside fluorometholone bulk crystals, which had been recrystallized from ethanol. Polymers used to help formulate the eye drops were also studied. Before x-ray analysis, each powder sample was ground in an agate bowl to avoid anisotropy. CuKα radiation (1.542 Å) was used, the x-ray output was 45 kV and 200 mA, and the running angle ranged from $2\theta = 5–35°$.

2.7. Synthesis of 20α- and 20β-dihydrofluorometholone

20α- and 20β-dihydrofluorometholone were synthesized following established methods (Norymberski and Woods, 1955; Okuda and Tanaka, 1990). As an initial step, fluorometholone (512 mg) was dissolved in methanol (200 mL), and sodium borohydride (127 mg) added to the solution on ice. After stirring for 1 h, acetic acid (250 μL) was added to stop the chemical reaction, followed by the addition of water to obtain precipitates. The precipitates were washed thoroughly with purified water and subjected to suction filtration using an isopore membrane filter (pore size: 0.05 μm) to obtain powders, which were a mixture of 20α- and 20β-dihydrofluorometholone. Confirmation of the identity of 20α- and 20β-dihydrofluorometholone was conducted by HPLC (Supplementary 1) and mass spectroscopy (Supplementary 2).

2.8. High pressure liquid chromatography (HPLC)

Solutions of 20α- and 20β-dihydrofluorometholone were subjected to HPLC using an ELITE LaChrom L-2000 series machine (Hitachi High-Technologies Corporation, Tokyo, Japan). The HPLC eluent was water: methanol at a ratio of 42:58, the flow rate was 1.0 mL/min at 35 °C, and the column used was a ODS-18 silica (YMC-Pack ODS-A: YMC Co. Ltd., Kyoto, Japan). The detection wavelength was $\lambda = 250$ nm, with a measurement time of 30 min. Retention times were 8.7 min, 12.0 min, 14.7 min, and 22.7 min, which corresponds with the internal standard (prednisolone), 20α-dihydrofluorometholone, fluorometholone, and 20β-dihydrofluorometholone, respectively (Supplementary 1).

2.9. Eye drop administration and HPLC

Ocular penetration of nanocrystal and microcrystal fluorometholone eye drops was investigated by measuring drug concentration in the aqueous humor of rabbits using HPLC. To achieve this 50 μL of the nanocrystal or microcrystal eye drop (0.1% w/w) was administrated into the conjunctival sac of one eye of a New Zealand white rabbit (3.0–3.3 kg, specific pathogen-free female, Kitayama Labs Co. Ltd., Nagano, Japan), and the eyelid gently held closed for 1 min to minimize outflow of the eye drop. No irritation reaction or toxic reaction was observed and all materials in the nano- and micro-eye drops at the amounts used were within approved tolerance levels endorsed by the Ministry of Health, Labor and Welfare of Japan. At pre-defined intervals (15 min, 30 min, 60 min, 120 min, and 240 min), and in separate experiments, aqueous humor (200 μL) was collected from anesthetized animals (ketamine 35 mg/kg + xylazine 5 mg/kg in intramuscular injection) using a 30 G needle syringe (Supplementary 3). Each collection of aqueous humor was conducted on successive weeks, giving the eye time to heal from the intraocular injection the previous week. After mixing 30 μL of aqueous humor with ethyl acetate (300 μL) followed by vortexing for 5 min, the upper phase of ethyl acetate (240 μL) was collected and placed in a centrifugal evaporator (CVE-3100; Eyela) for 30 min (35 °C, 1000 rpm), resulting in a residue. The residue was subsequently mixed with 60 μL of HPLC eluent (water:methanol ratio = 42:58) for HPLC analysis, with a sampling volume of 50 μL. After collection of the aqueous humor, anesthetic eye drops (oxybuprocaine hydrochloride 0.4% w/w) were applied for pain relief and antibacterial eye drops (levofloxacin 1.5% w/w) and ointment (ofloxacin 0.3% w/w) were administrated to prevent infection. Six eyes of six rabbits were used for each eye drop administration. All animals were managed in accordance with the rules of the Animal Experiment Committee of Osaka University, as well as with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The concentrations of 20α-dihydrofluorometholone and fluorometholone in the aqueous humor after the administration of fluorometholone nanocrystal or microcrystal eye drops were calculated using the calibration curves of 20α-dihydrofluorometholone and fluorometholone, respectively, where the peak areas in HPLC measurements were converted to drug concentrations. For statistical analysis, a Shapiro-Wilk normality test was used, and normality confirmed. The drug concentration in the aqueous humor is presented as the mean value ± 1.96 × SEM (standard error of the mean), which represents a 95% CI (Confidence Interval). Significance was then assessed using a Student's two-tailed *t*-test for samples of the aqueous humor that had received nanocrystal eye drops or microcrystal eye drops. A threshold of $p < 0.05$ was considered to be significant. All statistical calculations were performed using R programming language under parametric conditions.

2.10. In vitro solubility study of fluorometholone nanocrystal and microcrystal eye drops

The *in vitro* release of fluorometholone from fluorometholone nanocrystal and microcrystal eye drops was evaluated using the reverse dialysis bag method (Seidlitz and Weitschies, 2012). Briefly, 500 μL of simulated tear fluid (STF, NaCl 0.67%, $NaHCO_3$0.2%, $CaCl_2{\cdot}2H_2O$ 0.008%, pH adjusted to 7.4) (Li et al., 2013) was introduced into each of 6 dialysis bags (MWCO 12,000–14,000 Da, Spectrum Laboratories Inc., USA). Six dialysis bags were then immersed in a beaker containing 300 mL of STF for 12 h in order to achieve equilibrium. Following this, 100 μL of fluorometholone nanocrystal eye drops was carefully micropipetted into the beaker which was maintained at 37 °C with stirring at 100 rpm. Herein, over time, the dissolved fluorometholone outside of the dialysis bags entered the bags. At predetermined time points, one dialysis bag was withdrawn from the beaker and immediately replaced with an equal volume of STF (n = 6 at each time point). The concentration of fluorometholone in the withdrawn dialysis bag was quantified by HPLC in a similar manner to that used to determine the concentration of 20α-dihydrofluorometholone and fluorometholone in the aqueous humor. This analysis was similarly conducted for fluorometholone microcrystal eye drops. Dissolution velocity of fluorometholone

nanocrystal and microcrystal eye drops was arrived at by calculating the velocity gradient using immersion time points from 0 min to 15 min.

3. Results

3.1. Quality evaluation of fluorometholone nanocrystal and microcrystal eye drops

Nanocrystal eye drops (Fig. 2A) were observed to be more transparent than microcrystal eye drops (Fig. 2B). Optical transparency typically arises because of light scattering from dispersed colloidal particles, thus the more transparent nanocrystal formulation is a natural consequence of the smaller particle size. SEM further revealed that fluorometholone nanocrystals tended to have a rectangular morphology (Fig. 2C), whereas, microcrystals were more rod-like in shape (Fig. 2D). The mean size of fluorometholone nanocrystals in eye drops was found to be 201.2 ± 14.1 nm (s.d.) by a DLS analysis (Fig. 3A). The mean size of fluorometholone microcrystals in eye drops was found to be 9.24 ± 4.51 μm (s.d.), where SEM was used to measure the long axis of the rod-like microcrystals (n = 191) (Fig. 3B). The polydispersity indices of fluorometholone nanocrystal and microcrystal eye drops were 0.19 and 0.45, respectively. The average fluorometholone nanocrystal particle size was, therefore, approximately 45-fold smaller than the microcrystal particle size. The ζ-potentials of nanocrystal and microcrystal eye drops, measured using an ELSZ-1000, were − 13.9 mV and − 12.1 mV, respectively (inset: Fig. 3A and 3B), indicating that crystals in both eye drops were stably dispersed. It was also apparent that the stable dispersion of fluorometholone nanocrystal eye drops was maintained long-term, with limited crystal growth (216.2 ± 19.3 nm (s.d.), −11.3 mV, PI; 0.18) seen after storage of suspensions for 6 months at 10 °C (Fig. 4). Finally, we note that fluorometholone nanocrystal eye drops are suitable for sterilization via syringe filtration with pore sizes of 0.22 μm, if desired. The concentration of the drug after filtration, however, decreases to about 0.07% because some is retained by the filter membrane. Therefore, if one wants to obtain a 0.1% concentration of nanocrystal eye drops after 0.22 μm filtration it would be necessary to use a higher initial concentration prior to filtration. Filtered fluorometholone nanocrystal eye drops are stable for at least 43 days at room temperature (25 °C) (Supplementary 4), thus using a sterilization filter with a pore sizes of 0.22 μm will be of potential benefit for the eye drop manufacturing process because it simplifies the sterilization steps.

3.2. Powder x-ray diffraction

Powder x-ray diffraction patterns of fluorometholone nanocrystals (Fig. 5A) and microcrystals (Fig. 5B) were similar to those obtained from standard fluorometholone (Fig. 5C). Namely, all three samples displayed the same main diffraction peaks at $2\theta = 10.4$ and 15.3°. Patterns from the nanocrystal and microcrystal formulations contained additional peaks at $2\theta = 19.1$ and 23.3°, which were rarely observed or not seen at all in patterns from fluorometholone (Fig. 5C). Subsequent investigations discovered that these peaks came from a polymer used in the eye drops; specifically, from polyoxyethylene (200) polyoxypropylene glycol (70) (Fig. 5D). (The poloxamer that has the same CAS No. (i.e. 9003–11-6) as polyoxyethylene (200) polyoxypropylene glycol (70) gives rise to similar powder x-ray diffraction patterns, which provides confirmatory evidence that the peaks at $2\theta = 19.1$ and 23.3°come from polyoxyethylene (200) polyoxypropylene glycol (70) (Ali et al., 2010)). HPMC and PVP did not show any specific peaks at $2\theta = 19.1$ or $2\theta = 23.3°$, but did have broad halo peaks in these areas (Fig. 5E and 5F). This corresponds with previous powder x-ray diffraction analyses of HPMC and PVP in which both were reported to be amorphous (Katzhendler et al., 1998, de Villiers et al., 1998). Overall, the x-ray diffraction data indicate that fluorometholone nanocrystals and microcrystals in eye drop formulations have the same crystal structure as intact fluorometholone. Interestingly, the powder x-ray diffraction pattern obtained from fluorometholone nanocrystals (without the peaks from polyoxyethylene (200) polyoxypropylene glycol (70)) corresponds with that of fluorometholone nanocrystals prepared by a nano spray dryer technique, previously reported (Baba and Nishida, 2013), highlighting the consistency of the crystal structure across different modes of preparation. Since the crystal structure of fluorometholone nanocrystals and microcrystals is the same, if nanocrystal growth occurred the nanocrystals would develop into microcrystals. However, our data show that this tends not to happen, with crystal growth within nanocrystal eye drops not seen over extended periods.

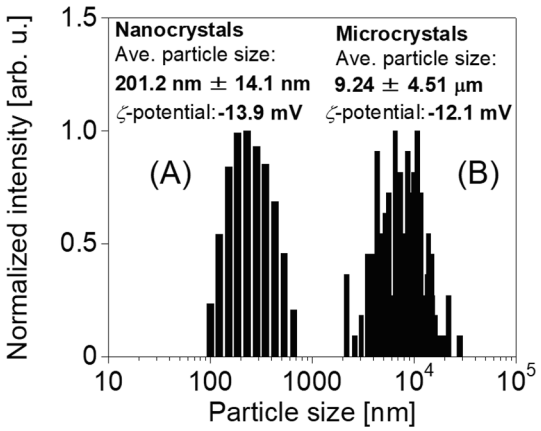

Fig. 3. DLS and ζ-potential measurements for fluorometholone nanocrystal (A) and microcrystal (B) eye drops.

3.3. In vitro solubility study of fluorometholone nanocrystal and microcrystal eye drops

Drug dissolution rate was calculated as a velocity gradient during the initial 15 min after the dissolution test started. This indicated that the dissolution rate of fluorometholone nanocrystals in eye drops was in the region of 1.6 times faster than the dissolution rate of fluorometholone microcrystals ($p < 0.01$) (Supplementary 5).

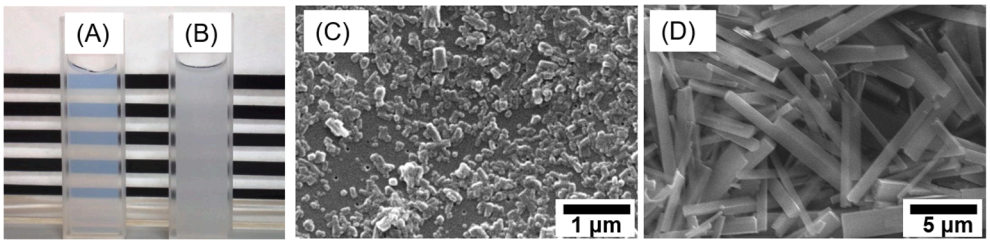

Fig. 2. The fluorometholone nanocrystal eye drops (A) are more transparent than the microcrystal (B) eye drops. On SEM, fluorometholone nanocrystals are small and rectangular in shape (C) whereas fluorometholone microcrystals are larger and rod-shaped (D).

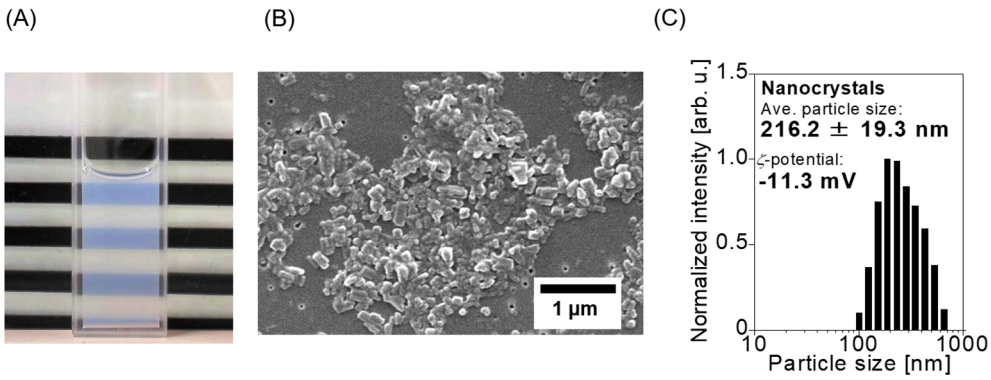

Fig. 4. After storage for 6 months at 10 °C, the transparency (A), crystal shape (B), and DLS and ζ-potential properties (C) of fluorometholone nanocrystal eye drops are essentially unchanged.

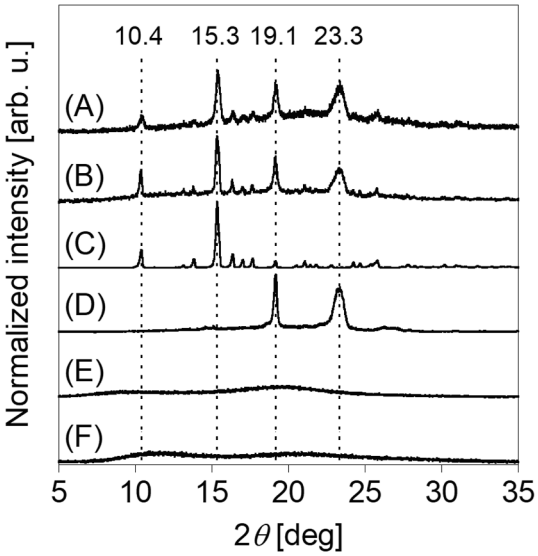

Fig. 5. Scans of powder x-ray diffraction patterns of fluorometholone nanocrystals (A), fluorometholone microcrystals (B), and fluorometholone crystals that were recrystallized from ethanol (C). Also shown in (D), (E) and (F) are the diffraction peaks from polyoxyethylene (200) polyoxypropylene glycol (70), hydroxypropyl methyl cellulose, and polyvinylpyrrolidone, respectively.

3.4. Drug concentration in the aqueous humor

Conventionally, to evaluate ocular drug delivery from fluorometholone eye drops, drug concentration in the aqueous humor, not the tear film, has been measured. Thus, the ocular penetration rate of fluorometholone eye drops into rabbit eyes *in situ* was investigated by measuring the concentrations of fluorometholone and dihydrofluorometholone in aqueous humor. Results of HPLC measurements at various timepoints after eye drop administration (15 min, 30 min, 60 min, 120 min, and 240 min) for the nanocrystal and microcrystal eye drops are shown in Supplementary 6. The results indicate that nanocrystal eye drops, when compared to microcrystal eye drops, attained a 2- to 6-fold higher average concentration when identified as the 20α-dihydrofluorometholone metabolite 15 min and 120 min after eye drop administration ($p < 0.05$–0.001; Fig. 6, summarized in Table 1). Moreover, 240 min after eye drop administration, 20α-dihydrofluorometholone was detected in the aqueous humor only in the case of nanocrystal eye drop administration. At the 30–60 min post-administration timepoints nanocrystal eye drop application resulted in a 2–3-fold higher average fluorometholone concentration in the aqueous humor, when compared to concentrations after microcrystal eye drop

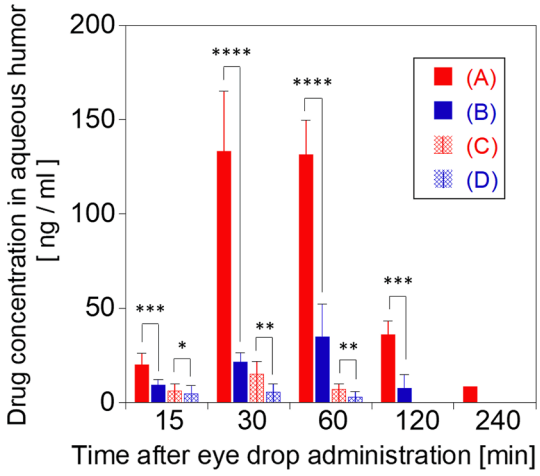

Fig. 6. Concentrations of 20α-dihydrofluorometholone (A, B) and fluorometholone (C, D) in the aqueous humor following application of fluorometholone nanocrystal eye drops (red) or microcrystal eye drops (blue); n = 6, * $P > 0.05$, ** $P < 0.05$, *** $P < 0.01$, **** $P < 0.001$.

Table 1
Concentration of 20α-dihydrofluorometholone in aqueous humor (ng/mL).

Eye drops	Time after eye drop administration				
	15 min (***)	30 min (****)	60 min (****)	120 min (***)	240 min
Nanocrystals	20.1 ± 6.1	133.2 ± 31.9	131.5 ± 18.2	36.0 ± 7.4	8.5
Microcrystals	9.3 ± 3.1	21.6 ± 5.0	34.9 ± 17.4	7.8 ± 7.2	–

Each data point: n = 6. Data are described as mean ± 1.96 × SEM (95% CI), apart from the 240 min nanocrystal data that are described as the mean of six samples. *T* test: *p* value is estimated using 95% CI of the mean difference between the nanocrystal and microcrystal eye drop administration; (***): $p < 0.01$, (****): $p < 0.001$.

administration ($p < 0.05$; Fig. 6, summarized in Table 2). In the early stages (i.e. after 15 mins) following eye drop use, no difference ($p > 0.05$) in the concentration of fluorometholone in the aqueous humor was seen when the nanocrystal and microcrystal formulations were compared. As described in the literature, 20α-dihydrofluorometholone is the main fraction in the aqueous humor, with fluorometholone present as a minor fraction (Okuda and Tanaka, 1990). In our investigations the 20β-dihydrofluorometholone metabolite was not detected in aqueous humor of rabbits throughout the whole 15–240 min period after eye drop administration, which is consistent with the findings of other

Table 2
Concentration of fluorometholone in aqueous humor (ng/mL).

Eye drops	Time after eye drop administration		
	15 min (*)	30 min (**)	60 min (**)
Nanocrystals	6.1 ± 3.7	15.4 ± 6.4	7.2 ± 2.7
Microcrystals	4.8 ± 4.1	5.4 ± 4.6	3.0 ± 2.8

Each data point: n = 6. Data are described as mean ± 1.96 × SEM (95% CI). *T* test: p value is estimated using 95% CI of the mean difference between the nanocrystal and microcrystal eye drop administration; (*): $p > 0.05$, (**): $p < 0.05$.

studies (Akura, 1987; Iqbal et al., 1993; Okuda and Tanaka, 1990).

4. Discussion

Fluorometholone has a strong anti-inflammatory effect, with an efficacy at least 25-fold higher than cortisone (Fairbairn and Thorson, 1971; Knopf, 1970) and nearly equal to that of dexamethasone (Gnad et al., 1973). In spite of its high strength, ocular hypertension caused by the steroidal side-effect of fluorometholone is low when compared to either dexamethasone or betamethasone (Fairbairn and Thorson, 1971; Jones and Rhee, 2006; Kersey and Broadway, 2006; Mindel et al., 1980). Fluorometholone mainly exists in the aqueous humor in its metabolized form, dihydrofluorometholone, which only weakly interacts with glucocorticoid receptors, and thus likely obviates any blockage of the trabecular meshwork ocular outflow facilities (Kato et al., 2006; Okuda and Tanaka, 1990). We contend that this metabolization of fluorometholone as it enters the eye has hitherto been overlooked as a reason for the low ocular hypertension-inducing properties of the drug, with previous concepts being based soley on a low levels of fluorometholone penetration through the cornea into the aqueous humor (Awan et al., 2009; McGhee et al., 1990). Despite the clinical importance of fluorometholone, only a few reports have discussed the ocular penetration of the drug and its conversion to its metabolized compound, dihydrofluorometholone (Akura, 1987; Iqbal et al., 1993; Kato et al., 2006; Okuda and Tanaka, 1990; Yamauchi et al., 1975). These have reported, for example, that the metabolization and entry into the anterior chamber of the rabbit eye of drug from a 0.1% fluorometholone eye drop suspension led to a 50 ng/ml concentration of 20α-dihydrofluorometholone in the aqueous humor 60 min after eye drop application (Okuda and Tanaka, 1990). To date, however, there have been no reports of the ocular penetration of fluorometholone and its metabolization to dihydrofluorometholone when applied as a nanocrystal formulation.

Here, we show that the fluorometholone nanocrystal eye drops we developed attained a 2- to 6-fold higher ocular penetration in the first 120 min after eye drop administration in rabbits, when compared with penetration of microcrystal eye drops. Moreover, the main fraction in aqueous humor was not fluorometholone, but the metabolite 20α-dihydrofluorometholone, and this was the case for both nanocrystal and microcrystal eye drop administrations. The nanocrystal eye drops also resulted in a comparatively longer duration release of drug into the aqueous humor as evidenced by the fact that 20α-dihydrofluorometholone was detected 4 hrs after eye drop administration in case of the nanocrystal formulation, with none detectable 4 hrs after microcrystal eye drops had been applied to the eye. Pharmaceutically, both crystal structure and particle size can affect drug bioavailability (Blagden et al., 2007; Junghanns and Muller, 2008). In the current study the foremost physicochemical distinction between the nanocrystal and microcrystal eye drops was particle size, rather than crystal structure, thus we conclude that a small particle size is the predominant reason for the observed differences in ocular penetration. Drug particle size is also an important factor when considering pharmacokinetics. On instillation of eye drops, drug particles from a suspension temporarily remain on the corneal surface and in the cul-de-sac. The pharmacokinetic behavior of the drug during that period is a balance between the supply rate (achieved by dissolution of the drug from the particles), the absorption rate into the cornea, and the elimination rate owing to drainage etc. (Hui and Robinson, 1986; Ueno et al., 1994). To achieve a satisfactory pharmacological effect, it is said that the dissolution rate must be faster than the clearance rate and equal to or higher than the absorption rate (Hui and Robinson, 1986; Ueno et al., 1994). The dissolution rate of a drug depends on particle size, with smaller particles generally being more soluble. It is known that nanocrystals have a higher dissolution rate than microcrystals (Junghanns and Muller, 2008), which, we contend, contributes to the nanocrystal eye drops having a higher intraocular penetration in our experimental system. This is supported by our measurements of drug dissolution rate, which shows that it is about 1.6 times faster for fluorometholone nanocrystals than for fluorometholone microcrystals.

Clinically, fluorometholone eye drops are valuable because their anti-inflammatory properties are augmented by the avoidance of glucocorticoids-induced ocular hypertension (Fairbairn and Thorson, 1971; Jones and Rhee, 2006; Kersey and Broadway, 2006; Mindel et al., 1980). Kato et al., 2006 investigated the binding activity of fluorometholone and the fluorometholone metabolites, 20α- and 20β-dihydrofluorometholone, to human glucocorticoid receptors using ^{3}H-dexamethasone displacement binding assays with the range of concentrations from 10^{-10} to 10^{-7} M *in vitro*. This found some concentration-dependent binding activity for 20α-dihydrofluorometholone (an inhibitory concentration of 60% at 10^{-7} M), but no binding activity for 20β-dihydrofluorometholone from 10^{-10} to 10^{-7} M. Fluorometholone, in contrast, displayed a comparatively strong concentration-dependent binding activity with an inhibitory concentration of 50% at 3.8×10^{-9} M (Kato et al., 2006). Nanocrystal fluorometholone eye drops are predicted to have a preferentially beneficial pharmaceutic effect in reducing inflammation and fibrosis of the cornea and ocular surface compared to microcrystal formulations for the reasons mentioned above. Meanwhile, the risks associated with the penetration of the drug into the aqueous humor are alleviated by the conversion in rabbit eyes to 20α-dihydrofluorometholone as the major fraction. This will result in a less deleterious steroid effect, compared with fluorometholone, on the trabecular meshwork within the anterior eye because of the weak binding activity to corticosteroid receptors. Indeed, in human eyes we know that fluorometholone is metabolized to 20β- dihydrofluorometholone rather than 20α-dihydrofluorometholone, so predicted steroidal activity in the aqueous humor would be even less (Iqbal et al., 1993). Nevertheless, care should be taken when using fluorometholone nanocrystal eye drops to treat the cornea and ocular surface because a minor fraction of fluorometholone appeared in the aqueous humor of rabbits, which was higher than that for conventional microcrystal eye drops. These considerations apply, too, to other forms of fluorometholone nano eye drops, where there is less knowledge about the relationship between fluorometholone and its dihydrofluorometholone metabolites and penetration into the eye. Limited amounts of fluorometholone reaching the aqueous humor would be useful when treating iritis (Yamauchi et al., 1979), for example, but careful control would be necessary to avoid excessive non-metabolized intraocular fluorometholone, and any benefits due to increased drug bioavailability should always be balanced with assessments of potential downsides. Indeed, high concentrations of fluorometholone in human eyes can lead to elevated intraocular pressure, which is an important consideration (Fairbairn and Thorson, 1971). The current study provides information about the physicochemical and pharmacokinetic properties of a new formulation of fluorometholone nanocrystal eye drops, their metabolization, and entry into the eye from eye drops. The therapeutic effects of fluorometholone nanocrystal eye drops will be investigated in other studies in the near future.

CRediT authorship contribution statement

Koichi Baba: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Noriyasu Hashida:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Motokazu Tujikawa:** Methodology, Formal analysis, Writing - review & editing. **Andrew J. Quantock:** Writing - original draft, Writing - review & editing, Visualization. **Kohji Nishida:** Conceptualization, Methodology, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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