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The in vitro evaluation of polyethylene glycol esters of hydrocortisone 21-succinate as ocular prodrugs

S.M. Foroutan [a], D.G. Watson [b,*]

[a] *School of Pharmacy, Shaheed Beheshti University of Medical Sciences, P.O. Box 1455-6153, Tehran, Iran*
[b] *Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow G1 1XW, UK*

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Abstract

The rate of hydrolysis of polyethylene glycol esters of hydrocortisone 21-succinate (H-PEGs) by a commercial esterase and by enzymes from ovine cornea was studied. Both the commercial esterase and the corneal enzymes rapidly hydrolysed the H-PEGs. Both lipophilicity and the PEG chain length had an influence on the rate of enzymatic hydrolysis, a significant decrease in enzymatic hydrolysis rate being associated with an increase in the polymer chain length. The in vitro penetration of H-PEGs across ovine cornea and sclera was assessed. In corneal penetration studies no prodrug was detected in the receiver phase while in the sclera penetration study both drug and prodrug were detected. Results from in vitro corneal and scleral absorption studies showed that the use of H-PEG400 increased the rate at which hydrocortisone diffused through excised ovine cornea compared to the use of hydrocortisone itself, particularly when the donor phase was removed after 15 min in order to mimic the situation in vivo. In addition, H-PEG200, H-PEG400, H-PEG600, H-PEG900 and H-PEG2000 gave a significant increase in the rate of diffusion of hydrocortisone + H-PEG through the sclera compared to hydrocortisone itself. Our results on ovine eye show that the rate of diffusion of hydrocortisone across the sclera is about six times higher than that in cornea and for the H-PEGs the scleral diffusion was 10–100 times higher than that observed in the cornea.

Keywords: Corneal and scleral penetration; Corneal enzymes; Hydrocortisone; Polyethylene glycol esters; Prodrug; Surface activity

1. Introduction

Previous studies have demonstrated that the derivatisation of drugs with polyethyleneglycol (PEG) chains is effective in prolonging the half-life of drugs in the body, changing the distribution of drugs within the body and protecting protein

* Corresponding author. Tel.: +44-141-548-2651; fax: +44-141-552-6443.
E-mail address: d.g.watson@strath.ac.uk (D.G. Watson)

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drugs from attack by proteases and from antibody attachment (Cecchi et al., 1981; Carini et al., 1990; Nucci et al., 1991; Nathan et al., 1994; Yamaoka et al., 1994; Bonina et al., 1995, 1996; Vincentelli et al., 1996; Bailon et al., 1997; Bentley and Chung, 1997; Nakaoka et al., 1997; Ouchi et al., 1997; Saifer et al., 1997). In the current study a number of polyethylene glycol esters of hydrocortisone 21-hemisuccinate (H-PEGs) were prepared with the aim of improving the ocular bioavailability of the drug and potentially other corticosteroids. In recent years PEGs have been considered as suitable promoieties for the synthesis of prodrugs (Ulbrich et al., 1986; Kohn et al., 1993; Bonina et al., 1996; Li et al., 1996; Vincentelli et al., 1996; Bentley and Chung, 1997; Fenton et al., 1997; Ouchi et al., 1997; Zhao and Harris, 1997). PEGs are widely used in pharmaceutical products because of their biocompatibility and lack of antigenicity and toxicity (Pang, 1993, 1997). Since PEGs are highly soluble in both water and a wide range of organic solvents, it would seem that the use of PEGs as promoieties for poorly water-soluble drugs used in ophthalmology could improve the hydrophilic–lipophilic balance of such drugs and increase their penetration through ocular tissues such as cornea and sclera.

In the first phase of the current work (Foroutan and Watson, 1997) a method for the synthesis of the H-PEGs was established and high-performance liquid chromatographic (HPLC) methods were developed for purification and analysis of these compounds. The H-PEGs were found to be highly water-soluble and their partition coefficients decreased with increasing chain length of the PEG promoiety. The present paper describes behaviour of H-PEGs in relation to their hydrolysis by a commercial esterase and by corneal enzymes and also studies their in vitro penetration through the cornea and sclera in comparison with hydrocortisone.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma-Aldrich, Poole, Dorset.

Esterase from porcine liver (19 units/mg) was obtained from Sigma-Aldrich. One unit will hydrolyse 1.0 mmol of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25°C.

2.2. Determination of surface activities and critical micelle concentrations of H-PEGs

At the beginning of each experiment fresh solutions of H-PEGs at different concentrations were prepared. The appropriate amount of each prodrug was dissolved in 2 ml of a fresh solution of 2% (w/v) boric acid and then adjusted to 5 ml in a volumetric flask with a solution of 2% (w/v) boric acid. The flasks were placed in an ultrasonic bath for a few minutes. Solutions with lower concentrations were prepared by diluting the concentrated solutions in 5-ml volumetric flasks. The clarity of each solution was judged by eye. In order to measure the surface tension of different concentrations of H-PEGs the Wilhelmy plate method was used and the measurements were made at room temperature. The surface tensions of water, methanol and acetonitrile (HPLC grade) were measured with the Wilhelmy plate as a control at the beginning of each experiment and were found to be close to the literature values (Weast, 1975). The plate was rinsed with fresh distilled water and dried between each reading.

2.3. Preparation of corneal homogenates and ovine eyes to be used in diffusion studies

Ovine eyes were collected from the slaughter house (Glasgow Abattoir Company) soon after the death of the animals and transported to the laboratory packed in ice. The eyes were used within 1 h of the death of animal and the cornea was carefully removed with a pair of ophthalmic scissors.

In order to prepare homogenates of freeze-dried cornea the corneas were placed in a suitable container which was loaded into a freeze-drier and the temperature control was lowered to −40°C. After complete freezing of the corneas the system was evacuated. After freeze-drying the corneas were powdered using a Waring Blender

in a cold room at 4°C, then homogenised in 1–2 ml of ice cold isotonic buffer solution (pH 7.4) and centrifuged at $3000 \times g$ for 5 min to remove the cellular debris. The resulting supernatant was diluted with isotonic buffer solution. Aliquots of the diluted supernatant (1 ml) were transferred to screw-capped vials. The Lowry method was used in order to measure the soluble protein content of corneal homogenates using bovine serum albumin as a standard.

In order to prepare cornea and sclera for penetration studies, eyes with no visual epithelial defects were used within 1 h of the death of animal. To facilitate dissection, the whole eye was placed in a holder. The cornea with a 2-mm ring of sclera was carefully removed with a pair of ophthalmic scisssors and mounted in the diffusion apparatus. During these preparatory manipulations, the epithelium was frequently moistened with glutathione bicarbonate Ringer (GBR) solution and great care was taken to avoid damage to the cornea. For scleral studies the sclera was removed from the eye once the choroid had been removed from the internal surface. An area was chosen that did not contain any trans-scleral perforations.

The hydration level of the cornea gives an indication of its condition. Ovine corneas which had been stored in GBR solution for 4 h were compared with fresh corneas. Any remaining sclera was removed and the trimmed cornea was weighed. The cornea was reweighed after freeze-drying at -20°C for 24 h and the hydration level could then be calculated.

2.4. Chromatography

For analytical HPLC a Thermo Separation Products model P100 pump was used linked to a Knauer UV detector. The system was fitted with a Rheodyne injection valve with a 20-μl loop. The detector was connected to a Hewlett-Packard integrator model HP 3395. A column (25 cm × 4.6 mm i.d.) packed with hypersil RP-18 with 5-μm particle size (Phenomenex, Macclesfield, UK) was used for analytical separations. Samples from the incubations were filtered of using 4-mm nylon membrane syringe filters (Burke Analytical, Glasgow) with a pore size of 0.45 μm.

The column was eluted at ambient temperature with acetonitrile–water (40:60) at a flow rate of 1.1 ml/min. The column effluent was monitored at 242 nm. Quantification of the H-PEGs and the hydrocortisone formed upon hydrolysis was carried out by measuring peak areas in relation to those of standards of known concentration chromatographed under the same conditions. Under these conditions the hydrocortisone produced by hydrolysis and the H-PEGs were separated while the H-PEG oligomers eluted as a single peak.

2.5. Studies of hydrolysis by esterase and corneal homogenates

The commercial esterase was dissolved in isotonic buffer (pH 7.4) to give a concentration of 0.1 mg/ml. Stock solutions of H-PEGs were prepared by dissolving the appropriate amount of prodrug in acetonitrile in order to obtain a concentration of 20 μmol/ml. All stock solutions were kept in screw-capped vials at 4°C. The reaction was initiated by adding 20 μl of a stock solution of the prodrug in acetonitrile to 1 ml of preheated esterase or corneal homogenate solution in a screw-capped vial at 37.5°C. The solutions were kept in a water bath at 37.5°C and at appropriate time intervals a 20-μl aliquot was taken and, after filtration using membrane syringe filter, analysed immediately by HPLC. All experiments were repeated three times for each H-PEG under the same conditions. Pseudo-first-order rate constants for the hydrolysis of the esters were determined from the slopes of linear plots of the logarithm of residual ester against time.

2.6. In vitro studies of the penetration of H-PEGs across cornea, sclera and corneal epithelium

A diffusion cell made of acrylic plastic(Fig. 1A) and consisting of a donor compartment (epithelial

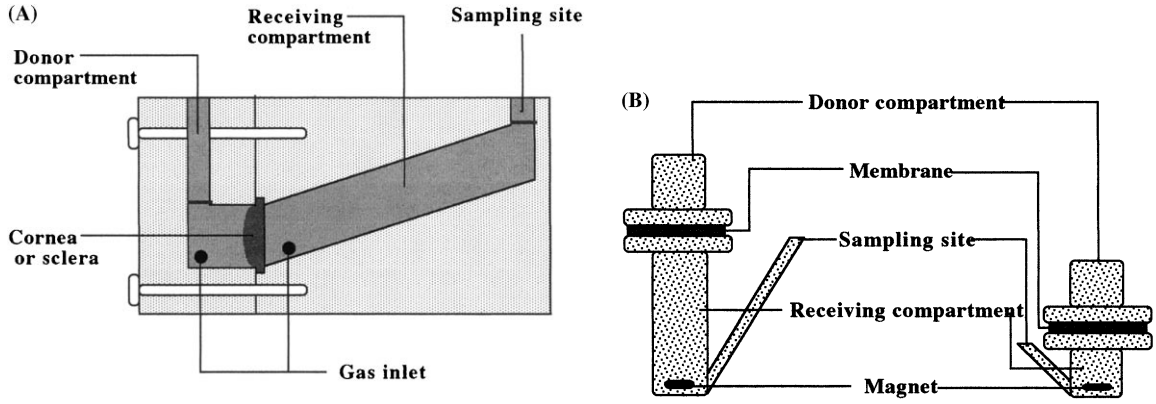

Fig. 1. (A) Perspex diffusion cell used for determination rate of diffusion of H-PEGs across the cornea and sclera. (B) Glass diffusion cells used for determination of the rate of diffusion of H-PEGs across the cornea.

side, volume 1 ml) and receiving compartment (endothelial side, volume 4 ml), in which the tissue was held in a vertical position, was used for corneal and scleral penetration studies. Mixing in each chamber was achieved by bubbling a mixture of 95% O_2 and 5% CO_2. The temperature was kept at 35°C by immersion in a circulating water bath. The area of the opening between compartments which was occupied by the cornea or sclera was 0.95 cm^2.

Glass diffusion cells (Fig. 1B) of two different sizes were made consisting of a 1ml donor compartment and 4ml or 1ml receiving compartments. Mixing in the receptor chamber was achieved by using a small magnetic stirrer bar. The area of the opening between compartments which was occupied by cornea was 0.785 cm^2.

After the cornea had been positioned in the perspex diffusion cell (Fig. 1A) 4 ml and 1 ml of preheated GBR solution (35°C, pH 6.85) were added to the receiver and donor compartments, respectively. The perfusion apparatus was placed in a bath pre-adjusted to maintain the solution temperature at 35°C. Before and during the experiments the GBR solution (Camber, 1985) was gassed with a mixture of 95% O_2 and 5% CO_2 using a gas bubble rate of 2 bubbles/s which achieved mixing in each chamber. A few minutes after the start of the experiment the GBR solution on the epithelial side was replaced with a O_2/CO_2 saturated GBR solution containing 5.44 μmol/ml of a particular H-PEG which was equal in molarity to a 0.2% (w/v) hydrocortisone suspension. Aliquots of 60 μl were withdrawn from the receiving side every 30 min for a period of 3.5 h and analysed by reversed-phase HPLC after filtration. Samples were analysed for remaining ester prodrug as well as for free hydrocortisone. The aliquot withdrawn from the receptor phase was replaced immediately with an equal volume of GBR solution. All experiments were repeated four times for each H-PEG under the same conditions.

In order to compare two types of diffusion cell the corneal penetration studies were also carried out using glass diffusion cells (Fig. 1B). After the cornea had been positioned in the apparatus, 4 ml and 1 ml GBR solution pH 6.85, preheated to 35°C, were added to the receiving and donor compartments, respectively. The perfusion apparatus was placed on a waterproof magnetic stirrer, in a bath pre-adjusted to maintain the solution temperature at 35°C. Mixing was achieved by using a small magnetic stirrer bar in the receiver compartment. A few minutes after the start of the experiment the GBR solution on the epithelial side was replaced with GBR solution containing 5.44 μmol/ml of a H-PEG which was equal in molarity to a 0.2% (w/v) hydrocortisone suspension. Aliquots of GBR solution (60 μl) were withdrawn from the receiving side every 30 min for a period of 3.5 h and analysed by reversed-phase HPLC after filtration. After a period of 3.5 h the prodrug solutions were removed carefully from the donor compartment and sam-

ples were taken from receiver compartment for another period of 3.5 h (total 7 h).

In order to study the release of the prodrugs which were absorbed into the corneal tissues over a short period of time, studies were carried out using small glass diffusion cells where the volume of the receiver compartment was 1 ml. The penetration studies were carried out as described above except that 200 μl of donor solution was used and was removed from the donor compartment after 15 min. Sampling and analysis was carried out as described above up to 4 h.

3. Results and discussion

The surface tensions or interfacial tensions (γ) of the H-PEGs were measured using the Wilhelmy plate method. The surface tension of the H-PEG solutions decreased as the concentration of the H-PEG was increased. The results show that the H-PEGs are surface active and that the surface tension of the air–water interface decreased with increasing concentration of compounds up to approximately 44 mM (H-PEG200), 9 mM (H-PEG400), 17 mM (H-PEG600), 18 mM (H-PEG900) and 32 mM (H-PEG2000). H-PEGs consist of a hydrophilic polar moiety (PEG) and a largely hydrophobic moiety (hydrocortisone) and therefore they accumulate at air–water interfaces. The plots of surface tension (γ) against the logarithm of the concentration of the H-PEGs in 2% (w/v) boric acid solution (pH 4.7; a potential formulation excipient) for H-PEGs are shown in Fig. 2 These results indicate that in very dilute solutions (< 0.7 mM) the surface activity is in the order H-PEG200 < H-PEG400 < H-PEG600 < H-PEG900 < H-PEG2000, i.e. the surface activity increases with increasing chain length of the PEG. The R.S.D. values of these measurements were all $< \pm 1.5\%$. Previous work showed that an increase in the length of the polyoxyethylene chain of non-ionic surfactants bonded to a constant hydrophobic chain length resulted in a decreased surface activity (Attwood and Florence, 1983a). Surfactant structure affects the surface activity and the hydrophobic groups of synthetic surfactants are often flexible alkyl groups whereas the

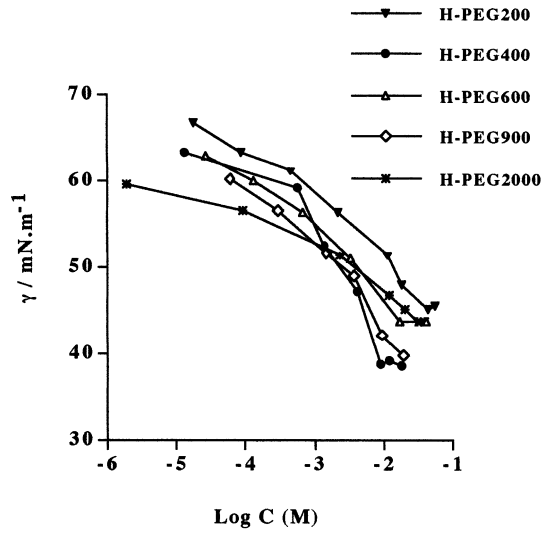

Fig. 2. Plots of the surface tension of solutions of H-PEGs in 2% (w/v) boric acid solution against concentration of H-PEG.

hydrophobic group of the H-PEGs is a rigid ring structure bearing some polar groups. The surface activity and aggregation properties of drugs have been discussed by Attwood and Florence (1983b). Differences in the structure of the alkyl group of surfactants causes aggregation to occur at varying concentrations. In this study, at higher concentrations turbidity was apparent; for example, H-PEG200 gave a milky solution in the range of 54.9–10.98 mM and H-PEG200 exhibited the lowest surface activity at all concentrations. No turbidity was observed at any of the concentrations of H-PEG400 studied. In the occular penetration studies, the concentration of the H-PEGs used was 5.44 mM, within the range where surface activity was still increasing with concentration for each compound and the solutions were clear. It can therefore be concluded that the H-PEGs were in a non-associated form in the occular test studies.

Porcine liver esterase was used to establish the in vitro susceptibility of the H-PEGs to enzymatic hydrolysis. Commercially available porcine liver esterase has the advantages of being stable, not requiring a co-factor and having the capability to hydrolyse a wide range of esters. The decrease in H-PEG concentration resulting from the hydroly-

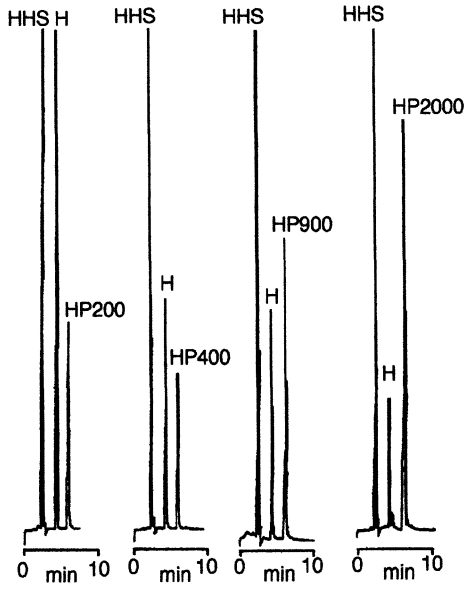

Fig. 3. HPLC chromatograms obtained after incubation of H-PEGs (0.04 μmol/ml) in the presence of porcine liver esterase at 37.5°C (pH 7.4.). The incubation times were 20 min for H-PEG200 and 90 min for H-PEG400, H-PEG900 and H-PEG2000. HHS, hydrocortisone 21-hemisuccinate; H, hydrocortisone; HP, H-PEG.

sis of H-PEG was monitored by reversed-phase HPLC. Fig. 3 shows chromatograms of some partly degraded solutions of H-PEGs obtained by HPLC analysis after incubation with esterase. As can be seen from the HPLC traces shown in Fig. 3, the disappearance of H-PEG was accompanied by the progressive appearance of free hydrocortisone and hydrocortisone 21-hemisuccinate. The chemical stability of H-PEGs in isotonic phosphate buffer pH 7.4 at 37.5°C was found to be adequate for the evaluation of enzymatic hydrolysis during the short time of the experiment. Pseudo-first-order rate constants, k_{obs}, for the enzymatic hydrolysis of the esters are shown in Table 1 and these were determined from the slopes of the linear plots of the logarithm of residual H-PEGs, against time. Regression coefficients (r) confirm the linearity of the fitted curves and consequently the first-order kinetics of the hydrolyses. The half-lives of the H-PEGs in phosphate buffer at pH 7.4 and 37.5°C are also listed in Table 1.

The enzymatic hydrolysis of the H-PEGs was also tested using the enzymes which are present in the cornea in order to assess the conversion of the prodrugs to the parent drug during passage across the cornea. Since the preparation of corneal homogenates can present some problems due to the tenacious nature of the outermost layer of the cornea, many authors have reported the possibility of using human plasma to assess the hydrolysis rates of ester prodrugs for different drug delivery systems. In the current work in order to assess the susceptibility of H-PEGs to bioconversion in the cornea a method of enzyme extraction was developed using freeze-drying. Because freeze-drying is a low-temperature process it is less destructive to the enzyme extract than homogenisation of fresh tissue.

As indicated in Table 1, in the absence of esterase at pH 7.4 and 37.5°C, half-lives exceeding 60 h were obtained for the H-PEGs while the half-lives in the presence of esterase were less than 2 h, thus demonstrating the strong catalytic effect of esterase enzymes. Fig. 4 shows the influence of the size of PEG chain of the H-PEGs on the rate of hydrolysis by esterase. Significant decreases in the enzymatic hydrolysis rate were observed with increasing PEG chain length and the associated decreasing lipophilicity of the H-PEGs. Chang and Lee (1983) reported that in an homologous series of 1- and 2-naphthyl esters there was a chain length which gave optimal hydrolysis and that this optimal chain length was influenced by the orientation of the ester side-chain with respect to the naphthalene nucleus. A peak rate of hydrolysis was exhibited by caproate and valerate esters for the 1- and 2-substituted naphthyl ester series, respectively. These findings suggested that the lipophilicity of the substrate alone did not control the rate of the substrate hydrolysis and that other physicochemical properties of the parent compound might affect the rate of hydrolysis of its esters. The size of the alkyl chain of esters has been reported to have an effect on esterase catalysed hydrolysis by Dixon and Webb (1979), who studied the reactivity of acetate and butyrate esters of C_1–C_8 alcohols with horse liver carboxyesterase. They found that a four-carbon alkyl chain length was hydrolysed most rapidly. In a

Table 1
Observed rate constants (k_{obs}) for the hydrolysis of H-PEGs by porcine liver esterase (2 units/ml) and enzymes extracted from ovine cornea (protein 3.69 mg/ml) and their half-lives in the presence and absence of corneal homogenates

Compound	Hydrolysis rate, $k_{obs} \pm$ S.D. min^{-1} (r)[a]		$t_{1/2}$ (h) at pH 7.4		
	In the presence of porcine esterase	In the presence of corneal esterase	Buffer	Porcine esterase	Corneal homogenate
H-PEG200	0.0263 ± 0.00006 (0.994)	0.031 ± 0.002 (1.000)	50.58	0.19	0.16
H-PEG400	0.0183 ± 0.00057 (1.000)	0.0216 ± 0.00057 (0.999)	54.71	0.28	0.23
H-PEG600	0.0086 ± 0.00057 (0.998)	0.0096 ± 0.00057 (1.000)	56.88	0.58	0.52
H-PEG900	0.00633 ± 0.00057 (0.989)	0.0063 ± 0.00057 (1.000)	59.92	0.79	0.79
H-PEG2000	0.003 ± 0 (0.999)	0.002 ± (0.999)	60.79	1.67	2.5

[a] Regression coefficient (r) of linear fit through the log of remaining prodrug.

series of *N*-substituted cytarabine ester prodrugs the enzymatic hydrolysis rate was dramatically increased within the aliphatic series from a methyl to an octyl moiety (Kawaguchi et al., 1996). A previous study of the hydrolysis of hydrocortisone aliphatic esters showed that the rates of ester cleavage varied with chain length and indicated that there was an optimum carbon number of about four to five required for a maximum hydrolysis rate and a dramatic decrease in esterase activity for acyl chains containing more than six carbon atoms. The combination of an enzyme and substrate may involve electrostatic forces in the case of charged groups, hydrogen bonding in the case of polar groups and van der Waals forces in the case of hydrocarbon chains. In the present case both hydrogen bonding and van der Waals forces are involved in the matching of the enzyme and substrate and the degree of matching is dependent on the chain length and hydrophilic–lipophilic balance of the hydrocortisone polymer conjugates.

By using a mixture of H-PEGs with different polyoxyethylene chain lengths it may, as a general principle, be possible to manipulate the pharmacokinetics of a drug to fit a particular situation. Fig. 5 shows the rate of enzymatic release of hydrocortisone from the solutions containing varying ratios of H-PEG200 and H-PEG900

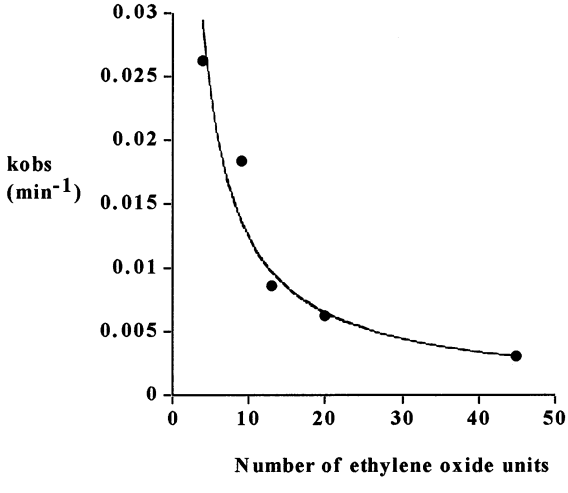

Fig. 4. Influence of the side-chain length of the H-PEGs on the rate of enzymatic hydrolysis by porcine liver esterase.

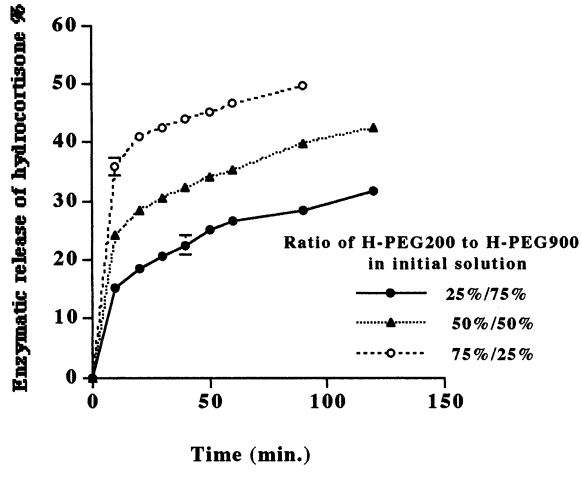

Fig. 5. Enzymatic release of hydrocortisone from the solutions containing mixtures with different ratios of 5.44 μmol/ml solutions of H-PEG200 and H-PEG900

where the total number of moles of hydrocortisone in conjugate form was kept constant. H-PEG200 hydrolyses much faster than H-PEG900 in the presence of esterase and incubation mixtures containing 1:3, 2:2 and 3:1 ratios of 20 μmol/ml solutions of H-PEG200 and H-PEG900 give different profiles for the release of free hydrocortisone from its H-PEG conjugates. As can be seen in Fig. 5, the amount of hydrocortisone released at each time is lowest for the solution containing H-PEG200:H-PEG900 in 1:3 mixture. An important difference between our study of PEG esters and previous studies using aliphatic moieties is that, for the H-PEGs, increasing the chain length of the PEG increases the aqueous solubility of the prodrug and it is possible to use any mixture of H-PEGs in aqueous solution; in the case of aliphatic chains there is a limited solubility in aqueous solution.

The H-PEGs can be hydrolysed by esterase so that either the bond to the oxygen on the 21-carbon in hydrocortisone is hydrolysed resulting in the release of hydrocortisone, or the ester bond between the succinic acid linkage and the PEG is hydrolysed resulting in the release of hydrocortisone 21-hemisuccinate which is a negatively charged ester. In the case of the incubations with corneal homogenate no hydrocortisone hemisuccinate was detected and all of the prodrug was converted to hydrocortisone, indicating that the mixture of corneal enzymes converts the hydrocortisone 21-succinate produced into hydrocortisone. The specific esterase enzyme of porcine liver cannot efficiently convert hydrocortisone hemisuccinate to hydrocortisone. The results agree with those obtained by other investigators. A study of the hydrolysis of derivatives of methylprednisolone in rhesus monkey serum and in human serum showed that negatively charged compounds were not hydrolysed by serum esterases while positively charged derivatives were cleaved relatively rapidly (Anderson and Conradi, 1987). Schottler and Krish (1974) investigated the hydrolysis of a variety of steroid hormone esters catalysed by non-specific carboxyl esterases from pig liver microsomes and found that charged esters such as the succinate esters of prednisolone and hydrocortisone were not hydrolysed. From these results the authors concluded that the absence of hydrolysis of anionic prodrugs such as 21-succinate esters in human serum and blood and their hydrolysis in vivo to the parent drug indicates that, while serum esterases may be important in cleaving some types of esters, esterases in other tissues must be involved in the bioconversion of anionic esters. It is also worth noting that in earlier work on the eye (McGhee et al., 1989) it was found that only prednisolone accumulated in human aqueous humor following topical application of prednisolone sodium phosphate to the eye and there was no evidence for prednisolone sodium phosphate surviving intact after passing through the cornea.

The degree of hydration of the cornea has an influence on drug penetration. It is not possible in in vitro experiments to control the hydration of the cornea at a normal level because, despite using isotonic solutions, the cornea still increases its hydration level. (Waltman and Hart, 1987). Schoenwald and Huang (1983) discarded the data in experiments in which the hydration levels of rabbit cornea increased to 83–92% and denoted them as being due to a damaged cornea. The hydration levels of ovine cornea which were determined using fresh eyes (removed no later than 2 h after the animal's death) was found to be $76.63 \pm 1.051\%$ ($n = 11$). The hydration level for normal cornea has been reported to be between 75% and 80% (Maurice and Riley, 1970; Waltman and Hart, 1987; Forrester et al., 1996). It is higher than that of other occular tissues such as the sclera which is about 70% hydrated. The hydration levels of pig (Camber, 1985) and rabbit (Saettone et al., 1996) cornea which were determined 0.5 h after the death of animals were reported to be $77.4 \pm 0.8\%$ and $80.4 \pm 1.1\%$, respectively. In the current study the hydration level of ovine cornea after 4 h in GBR solution was found to be $84.27 \pm 1.085\%$ ($n = 11$), while the hydration level of pig and rabbit cornea after 4 h in a diffusion apparatus in contact with GBR solution has been reported to be $78.4 \pm 0.7\%$ and $80.4 \pm 1.1\%$, respectively. The reason for a greater increase in the hydration level of ovine cornea in comparison with pig and rabbit cornea after 4 h in contact with GBR solution might be because of difference

in the species or a difference in the drying methods used. In the present study freeze-drying was used for drying the cornea whereas other investigators used an oven at 100–103°C for 6–12 h. Also the GBR solution in a clamped diffusion apparatus is only in contact with two sides of cornea but in the current study the cut edges of cornea also were in contact with the GBR solution. Corneal thickness increases with hydration and the diffusion coefficient for molecules passing through the cornea is inversely proportional to the barrier thickness. In the current study the results showed that the hydration level of sheep cornea showed very low variation ($\pm 1.37\%$) and even after 4 h contact with GBR solution the increase in hydration level of all corneas in the sample showed a variation of only $\pm 1.25\%$. Therefore it was possible to compare all the compounds in this study in terms of diffusion through the cornea while being confident of a lack of variation in corneal hydration.

The penetration of the H-PEGs through the cornea with time was determined using the method described in Section 2.6. The design of the perspex cell (Fig. 1A) was based on that of Camber (1985). The height of the solution in the receiving compartment is higher than that in the donor compartment and the consequent pressure difference gives some advantages. Firstly, the cornea is kept in place by the pressure difference and not by mechanical clamping which can induce damage to its different cell layers. Secondly, the higher water pressure on the endothelial side prevents the cornea from buckling; and thirdly, leakage between compartments is easily detected. Mixing in each chamber was achieved by bubbling a mixture of 95% O_2 and 5% CO_2 into both chambers while at the same time keeping the cornea in good condition. Using GBR solution in the bathing medium prolongs the ability of the cornea to maintain a constant thickness and allows the functioning of the full complement of ion transport systems. Following addition of GBR solution containing 5.44 μmol/ml of hydrocortisone in suspension and 5.44 μmol/ml of the different H-PEGs to the donor compartment, the hydrocortisone concentrations at various times were measured in the receiver compartment (endothelial side of cornea) using the HPLC method. Only a small fraction of H-PEG present in the donor compartment permeated through the cornea during 4 h and thus the H-PEG concentration in the donor compartment did not change significantly during the experiment. No prodrug was detected on the endothelial side of the cornea within the period of the experiments. The results showed that all the H-PEGs were hydrolysed by corneal enzymes during their passage across the cornea. The complete hydrolysis of the H-PEGs by the corneal enzymes during their passage across the cornea meant that the rate of penetration of prodrugs of hydrocortisone was equal to the rate of hydrocortisone release on the endothelial side of the cornea. Following a lag time of 50–90 min the rate of increase in the concentration of hydrocortisone in the receiving compartment was linear for all the compounds studied. The data points closely fit the least-squares regression line once a steady state has been reached. The rate constants for corneal permeation were calculated from slope of the linear portion of the plots. (Table 2) according to Schoenwald and Huang (1983) and Camber (1985). The linearity of the plots confirmed that the integrity of the cornea was maintained during the 4-h period of the experiments. In order to compare the different H-PEGs which have different molecular weights but which were used at the same molar concentrations, the data in Table 1 were calculated in μM

Table 2
Rate constants for corneal diffusion of hydrocortisone and the H-PEGs obtained using the perspex diffusion cells

Compound	$k \pm$ S.D. ($\mu M\ min^{-1}$)[a]	r[b]	Log D_{app}[c]
Hydrocortisone	0.041 ± 0.0067	0.994–1.000	−5.421
H-PEG200	0.025 ± 0.0017	0.992–0.994	−6.492
H-PEG400	0.071 ± 0.024	0.998–0.999	−6.036
H-PEG600	0.017 ± 0.0021	0.980–0.996	−6.669
H-PEG900	0.0053 ± 0.0025	0.992–0.993	−7.165
H-PEG2000	0.031 ± 0.0026	0.982–0.996	−6.398

[a] Rate constants for corneal permeation rates ± standard deviation ($n = 3$ for each experiment).
[b] Correlation coefficients which were used to identify the linear part of the rate curves.
[c] Logarithm of apparent permeation coefficient (cm s^{-1}).

min^{-1}. As can be observed in Fig. 6, H-PEG 400 appears to exhibit the fastest rate of penetration across the cornea in comparison with hydrocortisone and the other H-PEGs. However, the difference was barely significant based on $n = 3$ (one of the corneas exhibited a particularly low rate of penetration for the H-PEG 400); H-PEG900 has the slowest corneal penetration rate. In previous work the corneal transport characteristics of a series of different molecular weight polyethylene glycols showed that PEG polymers with polymer units longer than 12–14 began to fold, becoming globular in shape (Liaw and Robinson, 1992) and this decreased their corneal absorption. The slower rates of penetration observed for the H-PEGs containing PEGs with a molecular weight > 600 may arise from this. The corneal permeation of H-PEGs decreases with increasing molecular weight except in the cases of H-PEG400 and H-PEG2000. A study by Hämäläinen et al. (1997) on the corneal permeation of PEGs showed a decrease in permeation rate with an increasing molecular weight of PEG to a minimum at PEG800 and thereafter the permeation rate increased slowly with increasing molecular weight. In the present study H-PEG900 shows a minimum permeation rate since H-PEG2000 has a higher permeation rate. The effect of the molecular weight of H-PEGs on the corneal permeation rate in comparison with PEGs is only one factor because in addition to molecular weight the physical structure, hydrophilic–lipophilic behaviour and the rate of enzymatic hydrolysis of the H-PEGs by corneal enzymes are other factors which have an influence on corneal permeation rate. The partition coefficients for the H-PEGs were reported previously (Foroutan and Watson, 1997) and the log P_{obs} values were found to be as follows: H-PEG200, 1.55; H-PEG400, 1.07; H-PEG600, 0.62; H-PEG900, -0.14; H-PEG2000, too low to measure. In the current work it can be observed that the corneal permeation of the H-PEGs increased with increasing partition coefficient. In previous studies the optimum log P for corneal drug absorption has been reported to be in the range of 1–2 (Lee and Li, 1989) and 2–3 (Järvinen and Järvinen, 1996), although the corneal permeation of steroid prodrugs does not always correlate with their partition coefficient (Lee and Li, 1989). In the present case, H-PEG200 and H-PEG400 have a log P between 1 and 2 and show a higher corneal permeation in comparison with the other H-PEGs. The penetration of the H-PEGs through cornea was also assessed using the glass diffusion cells. The glass diffusion cells were used in current study for two reasons: firstly, to compare this type of simple cell with the perspex cell; and secondly, because it was easy to remove the donor phase in the one chambered glass diffusion cell after a certain time. The results for the penetration of hydrocortisone and the H-PEGs through the cornea obtained using the glass cell were similar to those obtained using the perspex diffusion cell. Small glass cells were designed and made in order to observe the release of drug from the cornea after removing the donor phase 15 min after its application. This was intended to mimic the real conditions of the eye in vivo because in vivo drugs do not remain on the surface of the cornea for long and are removed by tears and blinking within a few minutes. The volume of the receiver phase was reduced to 1 ml in order to detect the

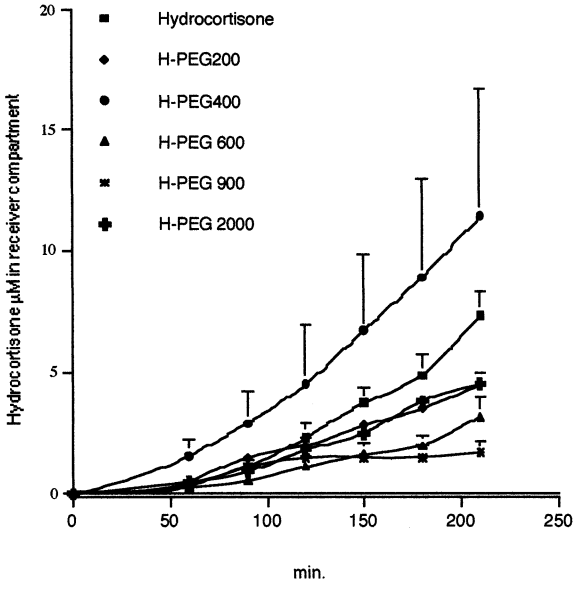

Fig. 6. Plot of the concentration of hydrocortisone accumulating on the endothelial side of the cornea versus time following the application of a solutions of hydrocortisone and H-PEGs on the epithelial side ($n = 3$ for each compound).

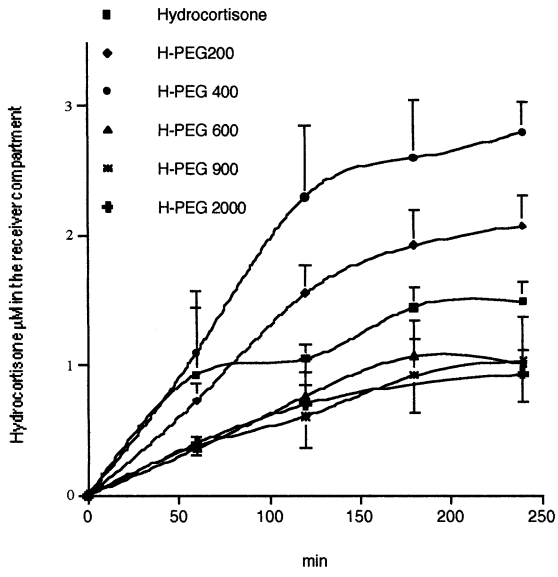

Fig. 7. Accumulation of hydrocortisone in the receiver compartment after removal of the donor phase at 15 min ($n = 4$).

smaller amounts of hydrocortisone accumulating there. In this experiment the amount of hydrocortisone which was released from the cornea into the endothelial side following removal of the solution of H-PEG from the donor compartment was measured up to 240 min. The results shown in Fig. 7 give a more clear-cut indication of the effects of conjugation to PEG on the corneal penetration characteristics of hydrocortisone. Both H-PEG400 and H-PEG200 permit penetration of significantly more hydrocortisone through the cornea than hydrocortisone itself up to 240 min.There is an indication that hydrocortisone is still accumulating in the receiver compartment 240 min after application of H-PEG200 and H-PEG400 whereas the accumulation from application of hydrocortisone suspension has leveled off. Thus the H-PEGs are providing a type of sustained release due to their altered diffusion characteristics in comparison with hydrocortisone. If the curve for the accumulation is examined more closely it can be see that while there is an initial rapid penetration of hydrocortisone it tails off very quickly. This is because hydrocortisone in solution probably partitions into the cornea more rapidly than it is replenished by dissolution of the suspended hydrocortisone. Thus the curve rises and this is followed by a lag phase while a true steady state is established as the rate of dissolution of hydrocortisone becomes the rate-limiting step. This effect is more marked in the experiment with the smaller glass cells where the volume of the donor phase was only 200 μl rather than 1 ml hence the initial pulse of hydrocortisone delivered from the hydrocortisone in solution will be smaller and thus not form as large a reservoir in the cornea. In vitro it would be most likely that most of the uptake from a suspension would be of the steroid already in solution and the particles of steroid in the suspension would be cleared rapidly from the surface of the eye. In the case of the H-PEGs the whole of the concentration of the drop is available for uptake as is the case with the steroid phosphates, which are used in eyedrops, but which are much less lipophilic than the H-PEGs.

The penetration of hydrocortisone and the H-PEGs across the sclera was measured in the perspex diffusion cell. Following addition of GBR solution containing 5.44 μmol/ml of hydrocortisone in suspension and solutions containing 5.44 μmol/ml of the H-PEGs to the donor compartment, the hydrocortisone and H-PEG concentrations in the receiver compartment were measured with time. Both drug and prodrug were detected on the endothelial side of the sclera. The rate constants for scleral diffusion were calculated from the linear portion of a plot of micromoles of total drug in the receiving compartment against time where the total drug refers to the sum of the micromoles of intact prodrug and hydrocortisone accumulating. The intact H-PEGs were detected in the receiver compartment because their penetration across the relatively thin sclera was faster than their rate of hydrolysis by the enzymes within the sclera. The correlation coefficients (r) for the linearity of accumulation of hydrocortisone + H-PEGs in the receiver compartment for each experiment confirmed that the integrity of sclera was maintained during the experiments. Fig. 8 shows the rate constants for the permeation of hydrocortisone and the H-PEGs across the sclera in comparison with cornea. The H-PEGs have a scleral penetration rate 1.5–4 times higher

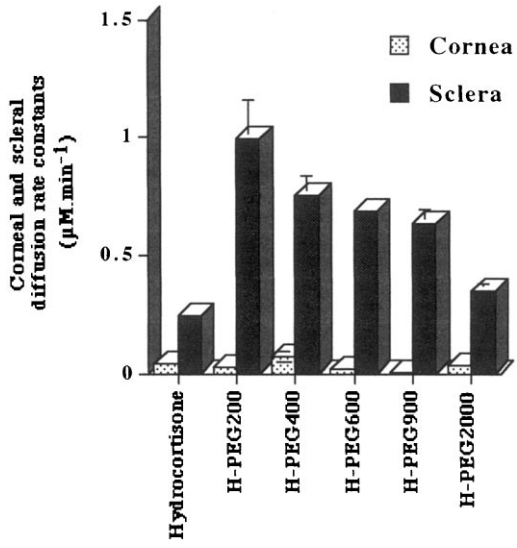

Fig. 8. The rate constants for the permeation of hydrocortisone and the H-PEGs across the sclera in comparison with those obtained for the cornea.

than hydrocortisone. The main reason for the difference is the low water solubility of hydrocortisone compared with the high water solubility of the H-PEGs. Also Fig. 8 shows that the rate of penetration of the H-PEGs through the sclera is much higher than their penetration through the cornea. This is probably due to the higher degree of hydrophilicity of the sclera in comparison with the cornea.

A study of the scleral permeation of PEGs in the rabbit cornea gave similar results to those obtained in the current study (Hämäläinen et al., 1997). In this study it was found that the diffusion coefficient of the PEGs decreased linearly with increasing molecular weight. In addition to molecular weight lipophilicity has also important role in scleral penetration. Hämäläinen and co-workers found in the rabbit that the scleral permeation of polyethylene glycols (MW between 238 and 942) was 4–8 times higher than corneal permeation. Our results on ovine eye show that the scleral diffusion of hydrocortisone is about 6 times higher than that in cornea and for the H-PEGs the scleral diffusion became 10–100 times higher than that observed in the cornea; therefore conjugation of PEGs to steroids could increase the scleral diffusion to a much greater extent than the corneal diffusion. For many years researchers have attempted to develop ocular drugs based on the premise that drugs enter the anterior chamber by penetration through the cornea. In recent years more definitive studies have been conducted to suggest that the high concentrations of drug found in iris/ciliary body tissue were a result of scleral absorption (Doane et al., 1978; Ahmed and Patton, 1985; Ahmed et al., 1987; Edelhauser and Maren, 1988; Hitoshi et al., 1989; Chien et al., 1990). Drug entering conjunctival tissue could be removed by systemic uptake from vessels embedded in that tissue but if it was able to pass that barrier then the evidence suggests that scleral resistance to penetration is less than the resistance from multilayered corneal tissue. If the ciliary process is the target site, direct access requires that drug must penetrate the conjunctiva, sclera, and finally the ciliary muscle to reach the ciliary process. Also the sclera contains the vessels which lead to the uvea and retina and, as an alternative to direct diffusion across tissue layers, provide a route by which drugs could reach the uvea and, importantly, the retina.

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