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Characterization of the Carrier-mediated Transport of Levofloxacin, a Fluoroquinolone Antimicrobial Agent, in Rabbit Cornea

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Abstract

The cornea presents a formidable barrier to drug penetration. The fluoroquinolone levofloxacin, which is an effective antimicrobial agent, has the potential to be used in the topical treatment of ocular disease. Thus, we sought to characterize how levofloxacin penetrates the cornea. To perform this characterization, we measured the time dependent permeation of levofloxacin across the isolated rabbit cornea using a diffusion chamber, and compared it with antipyrine fluxes.

Levofloxacin permeation into the receiver epithelial-side bathing solution (pH = 6·5) from the donor endothelial-side (pH = 7·4) reached 3·00 nmol cm^{-2} cornea after 2 h, whereas in the opposite direction permeation was 1·89 nmol cm^{-2} cornea. Based on the temperature-dependent effects on permeation, the calculated energy of activation for permeation, E_a, was 31·3 kcal mol^{-1}, whereas E_a for antipyrine, a marker of diffusion, was 11·0 kcal mol^{-1}. The transport of levofloxacin from epithelium to endothelium was concentration–dependent and had both a linear and saturable component. Evaluation of the kinetic parameters, J_{max}, apparent K_m and k_d showed that they were 38·78 pmol min^{-1} cm^{-2}, 3·83 mM and 0·0135 μL min^{-1} cm^{-2}, respectively.

These results, coupled with the fact that levofloxacin permeation reached a maximum value at pH 6·5, suggest that levofloxacin transport across the cornea is carrier mediated. However, at present, it cannot be ascertained whether such a system is localized in either the corneal epithelial or the endothelial layer.

Levofloxacin, a newly developed fluoroquinolone antimicrobial agent, is an optically active isomer of its racemate, ofloxacin. Levofloxacin is about twice as effective as ofloxacin in its antimicrobial action (Hayakawa et al 1986; Une et al 1988). Following instillation of levofloxacin to rabbit eyes, its concentration in the aqueous humour increases to a much higher amount than that of any of the other fluoroquinolones (Kawashima et al 1995; Sasaki et al 1995). As predicted from diffusion, the corneal permeation of the fluoroquinolones was found to correlate closely with their lipophilicity as evaluated by their partition coefficient (n-octanol/buffer; PC) (Fukuda & Sasaki 1995). However, there is evidence suggesting that transport is carrier mediated on corneal tissue. This is born out by noting that levofloxacin is an amphoteric moiety at physiological pH and has a pI at 6·75 (pK_{a1} = 5·5 for carboxyl group and pK_{a2} = 8·0 for piperazinyl group) and therefore a lower partition coefficient than at 7·5 (i.e. pH 6·5, PC = 0·33; pH 7·5, PC = 0·37, maximum: mean, n = 3, unpublished data). Nevertheless, it was shown in the pH range 4·5–8·5 that penetration from the tears into the aqueous humour in rabbit was greater at pH 6·5 than 7·5 (Kawazu et al 1996). Therefore, it is doubtful that levofloxacin permeates into corneal tissue only by passive diffusion. Our finding may be suggestive of a carrier-mediated mechanism for corneal transport of levofloxacin. That fluoroquinolone uptake or transport is carrier mediated is supported by findings in some other tissues and biological membranes (Prieto et al 1988; Okano et al 1990; Simanjuntak et al 1991; Griffiths et al

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1993, 1994; Hirano et al 1995a, b; Ohtomo et al 1996; Rabbaa et al 1996). However, there is no direct evidence for such a mechanism in the cornea.

We report here on our characterization of levofloxacin transport in rabbit isolated cornea. Our results suggest that there is a carrier-mediated component contributing to levofloxacin transport in corneal tissue.

Materials and Methods

Materials

Levofloxacin was kindly supplied by Daiichi Pharmaceutical Co. Ltd (Tokyo, Japan). Antipyrine was obtained from Wako Pure Industries Ltd (Osaka, Japan). The glass apparatus for the diffusion chamber (Permcell KH-5P) was obtained from Vidrex (Fukuoka, Japan). All other chemicals were commercial products of reagent grade.

Male, New Zealand albino rabbits (1·5–2·0 kg) were purchased from Kitayama Labs Ltd (Nagano, Japan). Rabbits were individually housed in cages in an air-conditioned room. Animals were maintained on a standard laboratory diet (RC-4, Oriental Yeast Co. Ltd, Tokyo, Japan) with water freely available.

In-vitro corneal permeation studies

Levofloxacin was dissolved in Hank's balanced salt solution (HBSS; consisting of (mM): 1·3 $CaCl_2$, 5·0 KCl, 0·3 KH_2PO_4, 0·8 $MgCl_2$, 138 NaCl, 0·3 Na_2HPO_4, 5·6 D-glucose and 10 MES for pH 5·5 and 6·5 or 10 HEPES for pH 7·4; osmolarity 315 mOsm kg^{-1}). In some experiments, 1 mM antipyrine was dissolved in permeated solution. Rabbits were killed with an overdose of pentobarbital sodium (Nembutal Sodium Solution, Abbot Laboratories, North Chicago, MI) administered via a marginal ear vein. The intact eye, along with the lids and conjunctival sac, was then enucleated. The cornea with its scleral ring was carefully cut out, and placed on the hemichambers of the diffusion monitoring system, which maintained the corneal curvature. The penetrant solution (4 mL HBSS, 1 mM levofloxacin, pH 6·5) was placed on the epithelial side. The endothelial side was drug-free (4 mL HBSS solution, pH 7·4). The apparatus was covered by a water jacket. Each chamber was gently stirred with a magnetic stirrer. At appropriate time intervals over 2 h, 200-μL samples were withdrawn from the receiver side, and replaced with an equal volume of HBSS.

Samples (100 μL) were assayed with a high-performance liquid chromatography (HPLC) system (Gulliver: Jasco Corporation, Japan) on a reversed phase TSKgel ODS-80T_S column (4·6 × 250 mm, particle size 5 μm, Tosoh, Japan). The mobile phase was a mixture of 0·01 M KH_2PO_4 (pH 3·0: adjusted by phosphoric acid) and acetonitrile (80 : 20, v/v). The flow rate was 1·0 mL min^{-1}. Retention times of drugs were monitored with a spectrofluorometer for levofloxacin (820-FP, Jasco Corporation; excitation wavelength 294 nm and emission wavelength 510 nm) and a UV spectrophotometric detector for antipyrine (UV-970, Jasco Corporation; 294 nm).

Data analysis of penetration studies

The apparent permeability coefficients (P_{app}) were calculated using equation 1:

$$P_{app} = V(dc/dt)/AC_0 \ (cm\ s^{-1}) \qquad (1)$$

where dc/dt is the flux across the corneal tissue (mMs^{-1}), V is the volume in the receiver chamber (mL), A is the surface area of the corneal tissue (0·6 cm^2), and C_0 is the initial concentration (mM) in the donor compartment. The flux across the corneal tissue describes the amount transported vs time and was calculated from the slope of the regression line obtained from the linear part of the curve. The energetic requirement of levofloxacin and antipyrine transport across the corneal tissue was calculated based on Arrhenius plots (eqn 2):

$$\log P_{app} = -[E_a/2.303R][1/T] + B \qquad (2)$$

where E_a is the activation energy, R is the gas constant, T is the absolute temperature, and B is the integration constant. A plot of log P_{app} against 1/T yields a straight line from whose slope (−E/2·303R) the activation energy can be determined (Grass & Robinson 1988). To estimate the kinetic parameters of saturable corneal transport of levofloxacin, the transport rate (J; cm s^{-1}) was fitted to equation 3, consisting of both saturable and nonsaturable linear terms, using the non linear least-squares regression analysis program, MULTI (Yamaoka et al 1981):

$$J = J_{max} \times S/(K_m + S) + k_d S \qquad (3)$$

where J_{max} is the maximum transport rate for the carrier-mediated process, S is the concentration of substrate, apparent K_m is the half-saturation concentration (Michaelis constant), and k_d is the first-order rate constant. In all cases, the data are expressed as the mean ± s.d. Statistical analysis was performed using Student's two-tailed *t*-test. A

difference between means was considered significant if $P < 0.05$.

Results

Effect of pH on permeation and directional permeation of levofloxacin

Levofloxacin penetration across the cornea is pH dependent in the rabbit in-vivo (Kawazu et al 1996). Varying the pH of levofloxacin ophthalmic solutions from 4·5 to 8·5 revealed maximum penetration at a pH of 6·5. The effects of pH on levofloxacin permeation were determined in the rabbit isolated cornea. When the pH of the donor side was varied from 5·5 to 7·4 and that of the receiver side was set at 7·4, levofloxacin penetration maximized at a pH of 6·5 (Table 1). This result agrees with that reported in-vivo (Kawazu et al 1996) and is suggestive of a carrier-mediated process for levofloxacin transport. To explore this possibility further, the directional levofloxacin permeation, which is from the epithelium to the endothelium and from the endothelium to the epithelium, was characterized. This was achieved by maintaining the donor side pH at 6·5 and the receiver side at 7·4. The results indicate that a pseudo-steady-state profile was eventually reached following a lag time of approximately 30 min (Figure 1). It should be noted that the endothelial- to the epithelial-side flux was consistently larger than the levofloxacin flux in the opposite direction. After 2 h, the amount of levofloxacin which had permeated from the endothelial to the epithelial side reached a level that was 59% larger than when levofloxacin penetration was measured over the same period in the opposite direction. Antipyrine flux, a marker for diffusion (Terasaki et al 1992), did not display net flux in either direction when the pH was 7·4 in both the epithelial- and the endothelial-side bathing solutions.

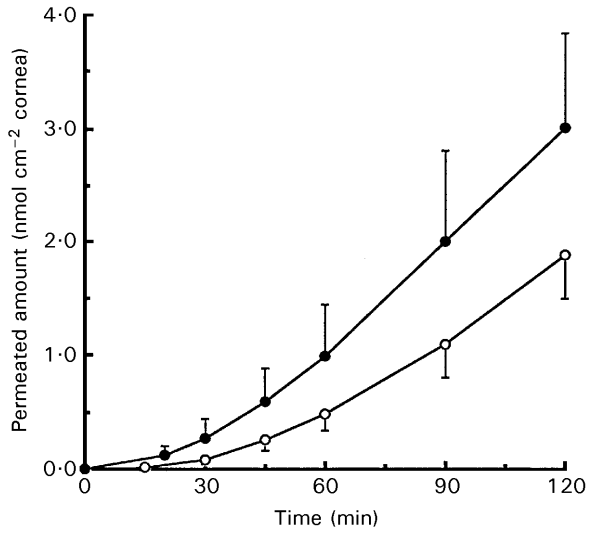

Figure 1. Time course of levofloxacin permeation across isolated rabbit cornea at 37°C. Levofloxacin was present at 1 mM. The donor- and receiver-side pH was 6·5 and 7·4, respectively. Each point represents the mean ± s.d. of three to four determinations. ● Endothelial to epithelial side, ○ epithelial to endothelial side.

Effect of temperature on levofloxacin permeation

The effects of lowering the temperature to either 25°C or 4°C on the epithelial- to the endothelial-side flux of levofloxacin and antipyrine were evaluated. The pH of the epithelial-side solution was 6·5 and the endothelial-side solution was 7·4, so as to enable calculation from Arrhenius plots of the relationship between P_{app} and temperature values for the E_a of levofloxacin and antipyrine. The effects of temperature change on P_{app} as well as the values for the E_a are shown in Table 2. Lower temperature markedly decreased the permeabilities of both levofloxacin and antipyrine. However, it is important to note that the P_{app} for levofloxacin declined much more with a decrease in temperature than that for antipyrine. For example, a lowering to 25°C decreased the P_{app} for

Table 1. pH, partition coefficient and kinetics of levofloxacin and antipyrine transport.

	pH	Partition coefficient	C_{max}[a] (μg mL^{-1})	Corneal permeability coefficients (P_{app}) ($\times 10^{-7}$ cm s^{-1})[b]	
				Epithelial side to endothelial side	Endothelial side to epithelial side
Levofloxacin	5·5	0·10	1·2	2·16 ± 0·38	3·84 ± 0·30*
	6·5	0·33	2·2	3·60 ± 0·46	6·07 ± 0·88*
	7·4	0·37[c]	1·9[c]	3·26 ± 0·36	4·32 ± 1·07
Antipyrine	7·4	1·70	–	59·9 ± 2·5	61·8 ± 8·0

[a]Maximum concentration in the aqueous humour after 3-topical instillations of 0·5% ophthalmic solution to rabbit eyes at 15 min intervals (Kawazu et al 1996).[b]The pH of the donor side was set at pH 5·5–7·4, and the receiver pH was set at pH 7·4. Corneal permeabilities were measured at 37°C. The values represent the mean ± s.d. of three to four determinations.[c]pH 7·5. *$P < 0.05$, significantly different from the opposite direction by Student's t-test.

Table 2. Effect of temperature on the corneal permeability and activation energy (E_a) for levofloxacin and antipyrine.

Temperature (°C)	Corneal permeability coefficients (P_{app}) ($\times 10^{-7}$ cms^{-1})[a]	
	Levofloxacin	Antipyrine
4	0·07 ± 0·05	0·67 ± 0·19
25	0·49 ± 0·27	23·73 ± 0·62
37	3·60 ± 0·46	49·23 ± 9·28
E_a[a] (kcal mol^{-1})	31·3	11·0

[a]Levofloxacin permeation was characterized with the donor and receiver sides having pH values of 6·5 and 7·4, respectively. Both levofloxacin and antipyrine were present at 1 mM. The data are shown as the mean ± s.d. of three to four determinations.

levofloxacin by 86% whereas the P_{app} for antipyrine fell by 52%. This larger decline for levofloxacin is consistent with an energetic requirement for carrier-mediated levofloxacin permeation. The involvement of a carrier-mediated levofloxacin permeation was indicated also by the fact that the E_a for levofloxacin was 31·3 kcal mol^{-1} whereas the E_a for antipyrine was 11·0 kcal mol^{-1}.

Concentration dependence

To understand the kinetics of levofloxacin permeation, the relationship between levofloxacin concentration over the range 0·25–50 mM and permeation from the epithelial to endothelial side was examined. The solution on the donor side had a pH of 6·5 whereas the pH was 7·4 on the receiver side. Figure 2 shows an Eadie-Hofstee plot of the results. In accordance with the suggestion that levofloxacin transport may be carrier mediated, levofloxacin penetration had a saturable concentration-dependent component. An evaluation of the kinetic parameters for this process revealed that: $J_{max} = 38{\cdot}78$ pmol min^{-1} cm^{-2} cornea; apparent $K_m = 3{\cdot}83$ mM; and $k_d = 0{\cdot}0135$ μL min^{-1} cm^{-2} cornea.

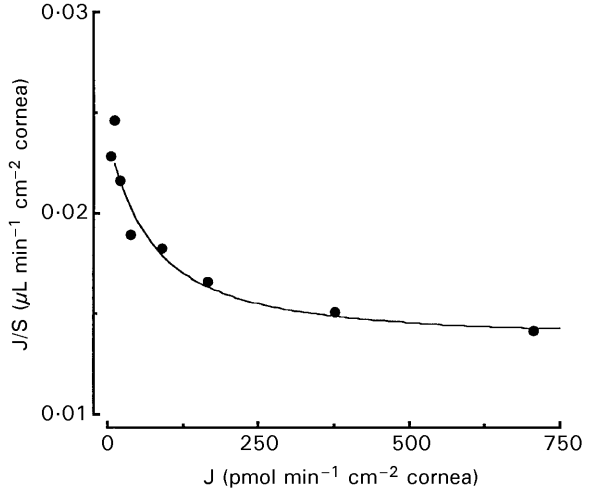

Figure 2. Eadie-Hofstee representation of levofloxacin permeation. The permeability of levofloxacin (0·25–50 mM) was measured at 37°C. Each point represents the mean ± s.d. of three to four determinations. The solid line for J was calculated by fitting the data to the Michaelis–Menten equation; $K_m = 3{\cdot}83$ mM, $J_{max} = 38{\cdot}78$ pmol min^{-1} cm^{-2} cornea, $k_d = 0{\cdot}0135$ μL min^{-1} cm^{-2} cornea.

Discussion

In this study there is substantive evidence for carrier-mediated transport of levofloxacin across the rabbit isolated cornea (from the epithelial to endothelial side). A stepwise reduction of the incubation temperature caused the permeation of levofloxacin to decrease more on a percentage basis than that of antipyrine. Measurements of the concentration dependence of levofloxacin fluxes revealed both a linear and a saturable component. Although the partition coefficient for levofloxacin is maximal at pH 7·5 (unpublished data), maximal levofloxacin permeation from the donor side occurred when the pH was 6·5. Another consideration pointing to a carrier-mediated uptake of levofloxacin is that the E_a of levofloxacin was significantly larger than those of several agents whose partition coefficients are similar to that of levofloxacin (water, butanol, and glycerol; e.g. approx. 5 kcal mol^{-1}) (Grass & Robinson 1988). We have shown that the transcorneal permeation in-vivo reached a maximum value at pH 6·5 (Kawazu et al 1996). Our findings suggest that carrier-mediated levofloxacin corneal transport resides in the cornea. Our results agree with those of Prieto et al (1988) for the intestinal absorption of ofloxacin. In that study, ofloxacin uptake could be fitted by a Michaelis–Menten process. In our study, the apparent K_m for corneal transport of levofloxacin was 3·83 mM, which corresponds to the K_m of 3·80 mM for intestinal absorption.

Levofloxacin fluxes from the endothelial- to the epithelial-side bathing solution were significantly larger than in the opposite direction. The efflux system for levofloxacin might exist on the rabbit isolated cornea and therefore this system might exert an influence on the carrier-mediated transport of levofloxacin from epithelium to endothelium. However, corneal tissue is composed of epithelium, Bowman's membrane, stroma, Descement's membrane and epithelium. It is not apparent from these studies whether the mechanism which mediates levofloxacin transport is localized in either the

corneal epithelium or endothelium. Further studies, focused on the levofloxacin transport by corneal epithelium or endothelium, are necessary. Studies in different tissues have suggested that facilitated levofloxacin transport is mediated by both a P-glycoprotein (Griffiths et al 1993, 1994), a cationic transporter (Okano et al 1990; Hirano et al 1995a, b; Ohtomo et al 1996; Rabbaa et al 1996), and a monocarboxylic acid transporter (Simanjuntak at al 1991). Also, we have demonstrated that levofloxacin transport in the cornea could be a consequence of monocarboxylic acid transport activity (data not shown).

We conclude that in addition to passive diffusion from corneal epithelium to endothelium, a specific carrier-mediated transporter in corneal tissue may account for the corneal permeation of levofloxacin.

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