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Original Article

EDTA decreases *in vitro* transcorneal permeation of fluconazole in phosphate buffer through excised sheep cornea

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Abstract

Introduction: According to the World Health Organization, corneal diseases are a major cause of vision loss and blindness, second only to cataract in overall importance. Fungal keratitis is a major blinding eye disease in Asia. In epithelia, calcium has been implicated in the maintenance of intercellular matrix and therefore may be a key factor determining the size of potential paracellular routes for drug transport. Although the effects of chelating agents such as EDTA on the permeability of inorganic and organic solutes have been well documented in other epithelia, as well as the corneal endothelium, no definitive studies examining the effects of these compounds upon corneal epithelia have been reported. **Materials and Methods:** The corneal permeation studies were conducted using freshly excised sheep cornea, mounted between donor and receptor chambers of an all glass-modified Franz diffusion cell, containing 11 ml of ringer bicarbonate (pH 7.4, 34°±1°C). At the end of the experiment, each cornea (freed from sclera) was weighed, soaked in 1 ml of methanol, dried overnight at 90°C and reweighed. From the difference in weights corneal hydration was calculated **Results:** Fluconazole ophthalmic solutions (0.2% w/v, pH 6.0) containing EDTA shows significant difference in P_{app} 1.51×10^{6} (cm/s) as compared to fluconazole ophthalmic solutions (0.2% w/v, pH 6.0) without EDTA showing 2.37×10^{6} (cm/s). **Conclusions:** Use of ethylene diamine tetraacetate as chelating agent in fluconazole ophthalmic solutions significantly decreased the corneal permeability of fluconazole.

Key words: Fungal keratitis, franz-diffusion cell, ocular, corneal permeability

INTRODUCTION

Fungal eye infections are rare.[1] The number of fungal infections has increased dramatically, and those involving the eye pose a serious problem and treatment challenge to practicing physicians.[2] Fungal keratitis is a major blinding eye disease in Asia.[3] Fungal keratitis is a serious and painful corneal inflammation that results from infection by a fungal organism.[4] The symptoms of fungal keratitis are blurred vision; a red and painful eye that does not improve when contact lenses are removed, increased sensitivity to light, and excessive tearing or discharge.[5]

Ocular conditions are usually treated by topical administration of drug solutions administered as eye drops into the lower cul-de-sac. These conventional dosage forms account for around 90% of the available ophthalmic formulations, mainly due to their simplicity and convenience.[6]

Drugs are commonly applied to the eye for a localized action. A major problem in ocular therapeutics is the attainment of an optimal drug conc. at the site of action. Poor bioavailability of drugs from ocular dosage forms is due to the precorneal loss factors, physiological and

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anatomical constraints.[7] Consequently, after instillation of eye drops, typically less than 5% of an applied dose reaches the intraocular tissues.[8] This forces the clinician to recommend a frequent dosing at an extremely high conc., and pulse type dosing results in several side effects of ophthalmic products.[9]

The last three decades have witnessed continued efforts aimed at improving the topical bioavailability of ophthalmic drugs. Investigations are being pursued along the following main lines:

1. Prolongation of the ocular residence time of the medication (vehicle approach, muco-adhesives);
2. Increase of the drug penetration characteristic (prodrug approach); and
3. Enhancement of the corneal permeability (enhancer approach).

The last approach, which consists of increasing transitorily the permeability characteristics of the cornea with appropriate substances, known as penetration enhancers or absorption promoters, bears a strict analogy with techniques aimed at facilitating drug penetration through the skin and different epithelia (buccal, nasal, intestinal, rectal etc.). However, the unique characteristic and great sensitivity of the corneal/conjunctival tissues impose great caution in the selection of enhancers with regard to consideration to their capacity to affect the integrity of epithelial surfaces.[10]

The synthetic bis-triazole antifungal compound fluconazole exhibits outstanding physical and pharmacokinetic properties. Fluconazole is a stable, water-soluble, bis-triazole antifungal that has low molecular weight, high bioavailability, good ocular penetration when used either systemically or topically, and low toxicity. It is potentially useful as a topical ocular agent. It is quite effective against *Candida* species.[11]

Fungal cell membrane synthesis is a multi-step process that involves the conversion of squalene to ergosterol. Fluconazole prevent the synthesis of ergosterol, a major component of fungal plasma membranes, by inhibiting the cytochrome P-450-dependent enzyme lanosterol demethylase (also referred to as 14 α-sterol demethylase or $P\text{-}450_{DM}$). Exposure of fungi to an azole causes depletion of ergosterol and accumulation of 14 α-methylated sterols. This interferes with the "bulk" functions of ergosterol in fungal membranes and disrupts both the structure of the membranes and several of its functions such as nutrient transport and chitin synthesis. The net effect is to inhibit fungal growth.[12]

MATERIALS AND METHODS

Materials

Fluconazole was obtained as a gift sample from Aurobindo Pharmaceuticals Limited Research Center, Mandal (A.P.). Sodium chloride, potassium chloride, magnesium chloride, calcium chloride, sodium bicarbonate, sodium dihydrogen orthophosphate dextrose and ethylene diamine tetraacetate sodium were purchased from Qualigens Fine Chemicals (Mumbai, India). All other chemicals purchased were of analytical grade and were used as received. Fresh whole eye ball of sheep were obtained from local butcher shop (Hisar, India), within half an hour of slaughtering the animal. The apparatus used in permeation studies was same as published elsewhere.[13]

Preparations of test formulations

Fluconazole ophthalmic solutions (0.2% w/v, pH 6.0) containing EDTA

Fluconazole 0.2% w/v ophthalmic solution in 0.0667M phosphate buffer (pH-6.0) made isotonic with mannitol and containing 0.01% EDTA was prepared.

Fluconazole ophthalmic solutions (0.2% w/v, pH 6.0) without EDTA

Fluconazole 0.2% w/v ophthalmic solution in 0.0667M phosphate buffer (pH-6.0) made isotonic with mannitol was prepared.

IN VITRO TRANSCORNEAL PERMEATION STUDY[14-16]

Corneal preparation

Whole eye ball of the sheep was obtained from local butcher shop within half an hour of slaughtering the animal, and was transported to the laboratory in cold (4°C) normal saline (0.9%) immediately. The corneal was carefully excised along with 2–4 mm of surrounding scleral tissues and washed with cold normal saline till free from proteins.

Permeation experiment

Fresh cornea was mounted by sandwiching the surrounding scleral tissue between clamped donor and receptor cells of modified version of Franz diffusion cell in such a way that its epithelial surface (apical) faced the donor compartment and endothelial surface faced the receptor compartment. Cell was placed on magnetic stirrer in holding position. The receptor compartment was filled with 11 ml of freshly prepared bicarbonate ringer solution (pH 7.4) and stirred using Teflon-coated magnetic stir bar. Drug solution (1 ml) was placed on the epithelial side of cornea in donor cell and stirring of the receptor fluid (jacketed with water at 34±1°C) was started. At appropriate intervals, 2 ml

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samples were withdrawn from the receptor compartment and withdrawn sample volume was replaced with equal volume of fresh bicarbonate ringer solution to ensure sink conditions. Withdrawn samples were analyzed spectrophotometrically (Varian-Cary 5000 UV-VIS-NIR) by measuring absorbance at λ_{max} of 260 nm. Each experiment was continued for about 2 h and was performed at least in triplicate.

At the end of the experiment, each cornea (freed from sclera) was weighed, soaked in 1 ml methanol, dried overnight at 90°C and reweighed. From the difference in weights, the corneal hydration was calculated.

Calculation of apparent permeability coefficient

The apparent permeability coefficient was calculated using the following equation:

$$P_{app.} = \Delta Q/\Delta t \times 1/ (A.C_0.60)$$

where $\Delta Q/\Delta t$ (μg/min) is the flux across corneal tissue, A is the exposed surface area of corneal tissue (0.786 cm^2), C_0 is the initial drug conc. (μg/ml) in the donor compartment and 60 is included to convert minutes to seconds. The flux across the cornea was determined from the slope of the regression line obtained from the linear part of the curve between the cumulative amount permeated (Q) vs. time (t) plot.

STATISTICAL ANALYSIS

Statistical calculation were done by one-way analysis of variance (ANOVA) followed by Dunnett's test. A P value <0.05 was considered significant.

RESULTS AND DISCUSSION

The first barrier to intraocular entry *via* the corneal and non-corneal pathways is the cornea and conjunctiva, respectively. These epithelial tissues are known to have tight junctions that act as a barrier in the paracellular spaces. Because of this barrier structure in the epithelia, insufficient drug may be absorbed after instillation. Thus, subconjunctival and intravitreous injections are generally used in ocular pharmacotherapy. These invasive methods may not be acceptable to many patients and could potentially increase the risk of infection. In order to obtain a simpler and more acceptable form of application, the development of effective instilled formulations would be a major improvement. A number of ocular penetration enhancers, including calcium chelator (EDTA) and bile salts, have already been investigated, and it was found that those enhancers increased the apparent permeability coefficient (Papp) of FITC-Dextran (MW: 4000, FD-4) from 2.9 to 15.5-fold in excised cornea and conjunctiva.[17] Penetration of molecules through the cornea is mainly limited by the outermost epithelial cell layer containing tight junctions. Absorption enhancers increase transitorily the permeability characteristics of physiological membranes and are used to facilitate drug penetration through the skin, the cornea and different epithelia (buccal, nasal, intestinal, and rectal). The use of absorption promoters was thought to be helpful in the formulation of ophthalmic preparations to increase therapeutic action of a drug or achieve an equivalent effect with a lower concentration of the active ingredients.[18]

Table 1 and Figure 1 compare the effect of EDTA on corneal permeation of fluconazole. EDTA, a known calcium-chelating agent has been shown to act on cell junction by interfering with calcium ions and altering intracellular integrity. EDTA also disrupts plasma membrane and consequently increases intercellular permeability.[19] EDTA has been reported to increase corneal absorption of various drugs through intact corneas.[20] However in our study use of EDTA in the formulation caused a significant decrease in apparent corneal permeability of fluconazole.

CONCLUSIONS

Use of ethylene diamine tetraacetate as chelating agent in fluconazole ophthalmic solutions significantly decreased the corneal permeability of fluconazole.

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Table 1: Comparative corneal permeation of fluconazole from 0.2% ophthalmic solutions containing EDTA in phosphate buffer (pH-6.0, 0.0667M)

	% Cumulative Permeation*			$P_{app.} \times 10^6$ (cm/s)	Relative $P_{app.}$	% Corneal Hydration
	60	90	120			
Phosphate buffer	21.11±0.45	24.03±0.95	25.95±1.05	2.37±0.09	1.0	80.70±0.37
Phosphate buffer + EDTA	12.20±0.76	15.16±1.43	16.50±1.60	1.51±0.15†	0.64	79.30±1.79

*Values are mean ± S.D. (n=3). † Significantly different ($p < 0.05$) as compared to control as determined by unpaired t-test.

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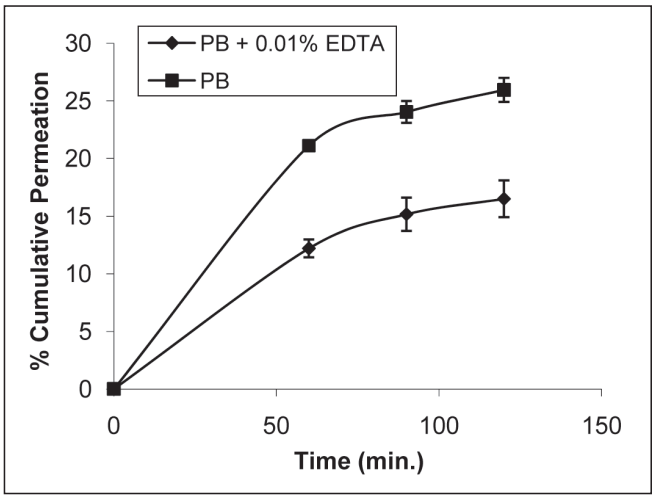

Figure 1: Effect of EDTA on *in vitro* transcorneal permeation of fluconazole

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