Permeability Characteristics of Novel Mydriatic Agents Using an in Vitro Cell Culture Model That Utilizes Sirc Rabbit Corneal Cells

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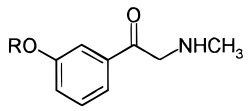
Abstract □ The purpose of this study was to evaluate the permeability characteristics of a previously reported in vitro corneal model that utilizes SIRC rabbbit corneal cells and to investigate the permeability of three novel esters of phenylephrone chemical delivery systems (CDS) under different pH conditions using this in vitro model. The SIRC rabbit corneal cell line was grown on transwell polycarbonate membranes, and the barrier properties were assessed by measuring transepithelial electrical resistance (TEER) using a voltohmmeter. The permeabilities of esters of phenylephrone CDS across the SIRC cell layers were measured over a pH range 4.0–7.4. The esters tested include phenylacetyl (**1**), isovaleryl (**2**), and pivalyl (**3**). The SIRC rabbit corneal cell line, when grown on permeable filters, formed tight monolayers of high electrical resistance with TEER values increasing from 71.6 ± 20.8 Ω·cm^2 at day 3 in culture to 2233.42 ± 15.2 Ω·cm^2 at day 8 in culture and remained constant through day 14 in culture. The transepithelial permeability coefficients (P_{app}) at pH 7.4 ranged from 0.58×10^{-6} cm/s for the hydrophilic marker, mannitol, to 43.5×10^{-6} cm/s for the most lipophilic molecule, testosterone. The P_{app} at pH 7.4 for phenylephrine was 4.21×10^{-6} cm/s. The P_{app} values and the lag times of the three esters of phenylephrone were pH dependent. The P_{app} for **1**, **2**, and **3** at pH 7.4 were 14.76×10^{-6}, 13.19×10^{-6}, and 12.86×10^{-6} cm/s, respectively and the permeabilities decreased at conditions below pH 7.4. The lag times at pH 7.4 were 0.10, 0.17, and 0.12 h for **1**, **2**, and **3**, respectively, and the values increased at lower pH conditions. The TEER values of SIRC cell line observed at day 8 to day 14 in the present investigation are similar to the resistance value reported for rabbit cornea (2 kΩ·cm^2). All the esters showed significantly ($p < 0.05$) higher permeabilities than phenylephrine at pH 7.4. The rate and extent of transport of the drugs across the cell layers were influenced by the fraction of ionized and un-ionized species and the intrinsic partition coefficient of the drug. The results indicate that the permeability of ophthalmic drugs through ocular membranes may be predicted by measuring the permeability through the new in vitro cell culture model.

Introduction

Phenylephrine hydrochloride (HCl) is an α-adrenergic agonist and is commonly used in routine ophthalmic practice as a mydriatic and vasoconstrictor agent. Several cases of adverse systemic reactions after topical ocular application of phenylephrine HCl have been reported and include severe hypertension, subarachnoid hemorrhage, ventricular arrhythmia, and possible myocardial infarction.[1−6] Therefore, it would be most desirable to design a drug that could be delivered to the eye compartments with the least possible systemic absorption and/or no systemic side effects. Previous reports have shown that, after topical application to the eye, esters of adrenalone but not adrenalone itself can be converted via a reduction−hydrolysis sequence to deliver adrenaline (epinephrine) only at the iris−ciliary body, the site of action.[7,8] This suggested that lipophilic ketones can be reduced in the iris−ciliary body. Accordingly, phenylephrone chemical delivery systems (CDS) (Figure 1) were designed to release the active species phenylephrine, by a "reductive−hydrolytic activation" mechanism, selectively to iris−ciliary body, thus avoiding the various systemic side effects. We recently reported the physicochemical properties of the novel compounds as a part of preformulation study.[9]



Phenylephrone CDS

1: R = -$COCH_2C_6H_5$ (phenylacetyl)
2: R = -$COCH_2CH(CH_3)_2$ (isovaleryl)
3: R = -$COC(CH_3)_3$ (pivalyl)

Figure 1—Structures of phenylephrone CDS.

Investigation of the absorption properties of a new drug moiety is also an important part in the preformulation process. The rate and extent of intraocular absorption and therapeutic effectiveness of topically applied drugs are dependent on the transport characteristics of ocular membranes, especially the cornea.[10−12] Characterization of the ocular penetration of drugs has been performed using hard-to-obtain ocular membranes from many animals. The establishment of a predictive method for the drug permeation using a cell culture model would be useful. In vitro cell culture models are of potential utility for some screening studies in which large quantities of corneas are needed. The SIRC rabbit cell line has been used by many researchers as a cellular model in studies of corneal physiology, immunology, and toxicology.[13−17] Hutak et al. recently developed an in vitro model for corneal permeability and reported that a single inoculation of SIRC rabbit corneal cells resulted in the formation of multiple epithelioid cell layers, with the number of layers increasing with culture time.[18] The objectives of the present study are to (a) evaluate the permeability characteristics of the SIRC rabbit corneal cell line system for potential use as an in vitro model for assessing drug permeability and (b) characterize the transport characteristics of three novel esters of the phenylephrone CDS using this model at varying pH conditions.

Materials and Methods

The SIRC cell line was obtained from American Type Culture Collection (ATCC no. CCL60; Rockville, MD). Earle's balanced salt solution (EBSS) without sodium bicarbonate, bovine calf serum,

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lactalbumin hydrolysate, yeast extract, trypsin, and gentamycin sulfate were obtained from Gibco BRL, Grand Island, NY. All other chemicals were of tissue culture grade. Plastic filter units, cell culture flasks (75 cm^2), and transwell inserts were obtained from Fisher Scientific, Houston, TX. D-[1-^{14}C]mannitol (mol wt 182.2) with a specific activity of 50.0 mCi/mmol and a radiochemical purity of 98% was purchased from Sigma Chemical Co., St. Louis, MO. [4-^{14}C]testosterone, having a specific activity of 57.0 mCi/mmol and a radiochemical purity of 98.2%, was purchased from Nycomed Amersham, Arlington Heights, IL.

Cell Culture—SIRC cells were cultured as previously described by Hutak et al.[18] The cells were maintained at 37° C in EBSS containing 10% bovine calf serum, 1760 mg/L lactalbumin hydrolysate, 570 mg/L yeast extract, 860 mg/L sodium bicarbonate, and 50 mg/L gentamycin sulfate in an atmosphere of 5% CO_2 and 95% relative humidity. The cells grown in 75 cm^2 flasks were passaged every seventh day of culture at a split ratio of 1:2. The cells were rinsed with 0.1% EDTA (pH 7.4), suspended using trypsin−EDTA in EBSS (37° C, 5 min), and transferred to two new flasks containing fresh growth medium.

In the present investigations, 0.5 mL of growth medium was placed in each well of the microwell plates, and the tissue culture inserts were placed in the wells. The cells, grown for 7 days in culture, were suspended in 50 mL of growth medium. A 0.15 mL aliquot of culture medium was placed into each filter insert, followed by the addition of 0.1 mL cell suspension. The total volume of media and cell suspension was predetermined by the filter capacity specifications. The volume of cell suspension added to the inserts was determined by the relative surface area of the microwell insert to the culture flask. The cell layers were given fresh growth medium on every seventh day of growth. One milliliter of fresh growth medium was placed into a new well. Following removal of the old medium, the insert was placed into this new well, and 0.1 mL of fresh medium was added to each insert.

Electrical Resistance Measurements—The permeability of the cell monolayers was determined by electrical resistance measurements using a voltohmeter. The culture media (0.6 mL and 0.1 mL to the apical and basolateral chamber, respectively) were added prior to electrical measurements. Transepithelial electrical resistance (TEER) values, obtained in the absence of cells (caused by the electrical system and the collagen-coated polycarbonate membrane), were considered as background. For each experiment, total electrical resistance values were corrected for background, which ranged from 20 to 40 Ω·cm^2, to obtain the TEER values of the cell monolayers.

Transport Studies—SIRC cell monolayers grown on collagen-coated polycarbonate filters (Transwell) for 8−10 days were used for transport experiments. On the day of the transport experiment, the culture media (0.6 mL and 0.1 mL to the apical and basolateral chamber, respectively) was replaced with EBSS containing 25 mM glucose. The pH of the transport medium was adjusted with HCl or NaOH. After equilibration at 37 °C for 30 min, TEER values were obtained prior to transport study. The integrity of each batch of cells was further tested by measuring the flux of radiolabeled mannitol (0.01 mM) in representative cell monolayers ($n = 3$). The transport of the novel mydriatic agents (phenylephrone CDS) across SIRC cell monolayers at pH 4.0, 5.0, 6.0, and 7.4 was determined in triplicate. In apical (donor compartment) to basolateral (receiver compartment) transport studies, each well in the 24 well clusters received 0.6 mL of transport medium that had previously been equilibrated at 37 °C. Transport medium (0.1 mL) containing the drug [phenylephrine, 0.1 mM; phenylephrone CDS, 0.1 mM; testosterone, 0.01 mM] was applied to the apical side. Samples (100 μL) were removed from the basolateral side at various times up to 180 min and replaced with the same volume of fresh transport medium. Aliquots of 20 μL were taken from the apical side at the beginning and end of each experiment.

Assay Method—The esters of phenylephrone (phenylacetyl **1**, isovaleryl **2**, and pivalyl **3**) were analyzed by HPLC (Isco pump, Model 2350; Model 484, waters) using an electrochemical detector. The detection potential was 1.15 V. A μBondapak C18 column with a similar 6 cm guard column and a mobile phase (pH 3.6) consisting of acetonitrile:acetate buffer:triethylamine (25:75:0.05) with a flow rate of 1 mL/min were used for the resolution of the compounds. Calibration curves were obtained by plotting the peak area as a function of drug concentration. The radioactive materials were analyzed by using a Beckman LS-3801 liquid scintillation counter.

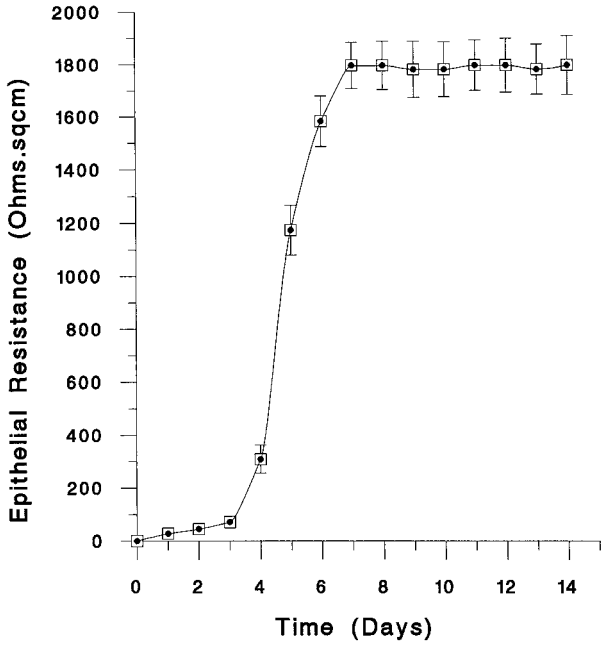

Figure 2—Transepithelial electrical resistance across SIRC cells grown on a polycarbonate membrane. Each value represents average (±SEM) of six experiments.

Data Analysis—Apparent permeability coefficients (P_{app}) of the three esters of phenylephrone and D-[1-^{14}C]mannitol and [4-^{14}C]testosterone were calculated using the following equation:

$$P_{app} = (1/AC_0)\,(dM/dt)$$

where dM/dt is the flux across the cell layers (μg/hr), A is the surface area of the membrane (0.33 cm^2), and C_0 is the initial drug concentration (μg/mL) in the donor compartment at $t = 0$. Flux per unit surface area ($1/A \times dM/dt$) was determined from the slope of linear portion of the cumulative amount permeated per unit surface area vs time plot. The lag time was also determined from this plot by extrapolating the linear portion to the abscissa. The results of experiments performed in triplicate are presented as mean ± SD. Statistical differences between the novel compounds in the amount permeated at each time point, and the means were determined by one-way analysis of variance (ANOVA). The criterion for statistical significance was $p < 0.05$.

Results and Discussion

Transepithelial Electrical Resistance Measurements—Measurement of electrical resistance across the cellular layers is a convenient and relatively sensitive measure of the integrity and permeability of the layer. These measurements reflect predominantly the resistance across the tight junctions and not the cell membranes.[19−23] The transcellular electrical resistance of the SIRC rabbit corneal cell layers increased with time up to the eighth day of culture with a resistance of 2233.42 ± 15.2 Ω·cm^2 and remained constant through the 14th day of culture (Figure 2). The electrical resistances shown by the SIRC cell line are slightly higher than the transepithelial resistances across the corneal membranes (2 kiloohms·cm^2).[24] However, methods used to derive resistance values from cell monolayers and excised tissue are different, and therefore comparisons should be considered with caution. The resistance values shown by the cell layers used in the flux experiments are slightly lower due to effects of media changes during washing. The TEER values were constant

Table 1—The Flux and P_{app} Values of D-[1-^{14}C]Mannitol and [4-^{14}C]Testosterone across SIRC Cell Layers at Different pH Conditions[a,b]

	pH 4.0	pH 5.0	pH 6.0	pH 7.4
		Mannitol		
flux (ng/cm^2 hr)	4.03 ± 0.08	3.87 ± 0.11	3.86 ± 0.21	3.80 ± 0.22
$P_{app} \times 10^6$ (cm/s)	0.62 ± 0.02	0.59 ± 0.01	0.59 ± 0.02	0.58 ± 0.02
		Testosterone		
flux (ng/cm^2 hr)	462.8 ± 12.6	459.3 ± 16.8	451.9 ± 14.7	456.5 ± 18.1
$P_{app} \times 10^6$ (cm/s)	44.1 ± 1.2	43.8 ± 1.6	43.1 ± 1.4	43.5 ± 1.8

[a] Values are means ± SEM of at least three experiments. [b] The values were obtained from the permeation studies performed up to 120 min at pH 4.0 and 5.0 and up to 150 min at pH 6.0 and 7.4.

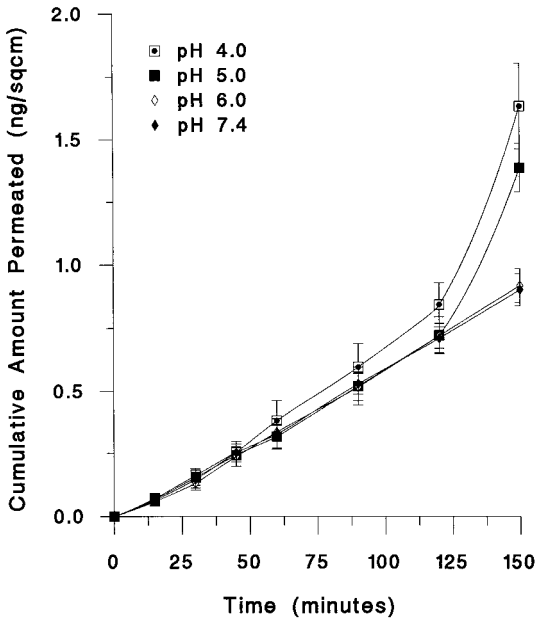

Figure 3—Apical to basolateral transport of mannitol across SIRC cell layers at different pH conditions. Each value represents average (±SEM) of at least three experiments.

throughout the transport studies at pH 6.0 and 7.4, but the values decreased when exposed to pH 4.0 and 5.0 after a period of 120 min.

Transport Studies—The permeability of the hydrophilic marker, D-[1-^{14}C]mannitol, was determined to further characterize the SIRC cell monolayers regarding its integrity as a molecular barrier. Mannitol, a molecule which is transported predominantly via the paracellular pathway, showed low permeabilities (0.58×10^{-6} cm/s) (Table 1) across the SIRC cell layers confirming that the cells formed tight junctions when grown on polycarbonate membranes. The permeability of mannitol across the SIRC cells was less when compared to the permeability across isolated corneal membranes.[25] Figure 3 shows the cumulative amount of mannitol permeated over time at different pH conditions. At pH 4.0, the mannitol permeability at 120 min was 0.62×10^{-6} cm/s and later increased to 2.1×10^{-6} cm/s. Similarly, the P_{app} values at pH 5.0 increased from 0.59×10^{-6} cm/s to 2.02×10^{-6} cm/s after 120 min. These differences in the permeabilities of the paracellular marker over time (and decrease in the resistance values) might be due to the damage caused to the surface layers of the SIRC cells when exposed to low pH conditions.

The gradual and reproducible development of resistance across the SIRC cells, when grown in culture medium, allowed study of the permeability characteristics of a group of novel mydriatic agents, esters of phenylephrone, and the lipophilic marker, testosterone. The highly lipophilic molecule, testosterone, is transported via the transcellular pathway. Testosterone exhibited a high permeability (43.5×10^{-6} cm/s) in the SIRC model at pH 7.4 (Table 1). Figure 4 illustrates the apical to basolateral transport of the phenylephrone esters, mannitol, phenylephrine, and testosterone across SIRC cell layers at pH 7.4.

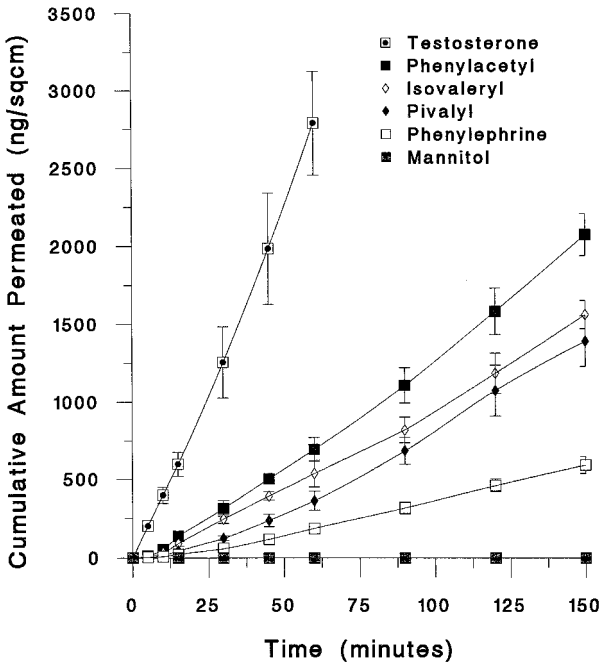

Figure 4—Apical to basolateral transport of testosterone, phenylephrone CDS, phenylephrine, and mannitol across SIRC cell layers at pH 7.4. Each value represents average (±SEM) of at least three experiments.

Table 2—The Flux, P_{app}, and Lag Time Values of Phenylephrone Esters across SIRC Cell Layers at Different pH Conditions[a,b]

	flux (μg/cm^2 h)	$P_{app} \times 10^6$ (cm/s)	lag time (h)
		Phenylephrine	
pH 4.0	0.33 ± 0.04	3.20 ± 0.08	0.51 ± 0.05
pH 5.0	0.35 ± 0.03	3.42 ± 0.10	0.49 ± 0.03
pH 6.0	0.40 ± 0.05	3.91 ± 0.26	0.31 ± 0.04
pH 7.4	0.43 ± 0.02	4.21 ± 0.34	0.28 ± 0.02
		Phenylacetyl Ester	
pH 4.0	1.18 ± 0.03	10.67 ± 0.28	0.39 ± 0.03
pH 5.0	1.22 ± 0.02	11.09 ± 0.31	0.30 ± 0.01
pH 6.0	1.41 ± 0.02	12.83 ± 0.21	0.19 ± 0.03
pH 7.4	1.62 ± 0.08	14.76 ± 0.61	0.10 ± 0.02
		Isovaleryl Ester	
pH 4.0	1.04 ± 0.02	10.08 ± 0.19	0.44 ± 0.02
pH 5.0	1.11 ± 0.02	10.77 ± 0.16	0.35 ± 0.02
pH 6.0	1.25 ± 0.03	12.17 ± 0.25	0.23 ± 0.03
pH 7.4	1.36 ± 0.05	13.19 ± 0.42	0.17 ± 0.01
		Pivalyl Ester	
pH 4.0	0.97 ± 0.01	9.51 ± 0.15	0.47 ± 0.03
pH 5.0	1.04 ± 0.02	10.14 ± 0.19	0.39 ± 0.01
pH 6.0	1.19 ± 0.01	11.65 ± 0.11	0.17 ± 0.02
pH 7.4	1.31 ± 0.04	12.86 ± 0.38	0.12 ± 0.02

[a] Values are means ± SEM of at least three experiments. [b] The values were obtained from the permeation studies performed up to 120 min at pH 4.0 and 5.0 and up to 150 min at pH 6.0 and 7.4.

The physicochemical properties of the diffusing solute and the physiologic function of the cell layer involved are the important factors affecting the transport rate. The phenylephrone esters had molecular weights 285.7−305.6, pK_a's 7.19−7.21, and log P values ranging from 1.92 to 2.35.[9] As shown in Table 2, the permeabilities of all the esters were significantly higher than that of mannitol ($p < 0.05$), indicating that the compounds might be permeat-

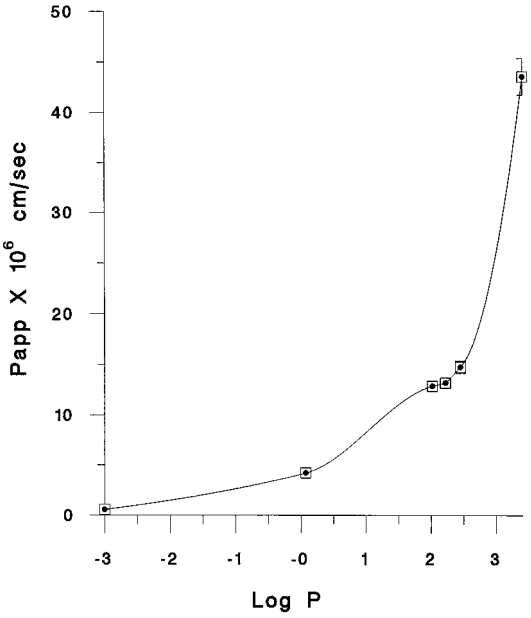

Figure 5—Relationship between apparent permeability coefficients of the various drugs and their respective log octanol–water partition coefficients at pH 7.4. From left to right, the compounds are mannitol, phenylephrine, pivalyl, isovaleryl, phenylacetyl esters of phenylephrone and testosterone. Each value represents average (±SEM) of at least three experiments.

ing via the transcellular pathway. Hamalainen et al. reported that molecules having a molecular weight less than 500 permeate through paracellular route.[26] Since all the compounds under investigation have low molecular weight, the paracellular route can also be considered as a pathway for permeation.

The permeabilities of the phenylpehrone CDS ranged from 12.86×10^{-6} cm/s to 14.76×10^{-6} cm/s at pH 7.4 and were found to be 3-fold higher compared to phenylephrine (4.21×10^{-6}). The rank order of permeation of the esters at any given pH was in accordance with their lipophilicities (**1** > **2** > **3**).[27] Phenylacetyl ester (**1**), which is the most lipophilic, exhibited a higher permeability compared to isovaleryl (**2**) and pivalyl (**3**) esters. Figure 5 illustrates the relationship between log octanol/water partition coefficients and transport rates of the compounds.

Permeability of all the three compounds was markedly dependent on the pH of the transport medium and also on the resistance of SIRC cell layers. The change in pH will affect the degree of ionization of the drug molecule if the drug has pK_a-value in that specific region. At pH 6.0 and pH 7.4, the esters showed significant differences in their permeation at all time points after 15 min ($p < 0.05$) but at pH 4.0 and pH 5.0 the differences in the amount permeated were found significant ($p < 0.05$) at time points after 45 min. At pH 6.0 and 7.4, the P_{app} value of phenylacetyl ester was significantly different from isovaleryl and pivalyl but at pH 4.0 and 5.0, only phenylacetyl and pivalyl esters showed significant difference in their P_{app} values. At higher pH conditions, the compounds under investigation predominantly exist in un-ionized form, causing an increase in diffusion through transcellular pathway resulting in higher permeabilities. The results showed decrease in permeabilities and increase in lag times of the compounds at lower pH conditions. One possible reason for the lag times might be due to the hydrolysis of the esters during the transport studies which are undetectable in the initial time points. Compound **2** showed higher lag time compared to **1** and **3** because of its higher rate of hydrolysis.[9] It is also possible that the lag times may be due to the hydrogen bonding between the compounds and the structures within the intercellular space that could impede molecular movement.[28]

The epithelia are negatively charged and are selective to positively charged solutes at physiological pH or pH above the isoelectric point (pI). Below the pI the reverse was observed.[29,30] Compounds used in this study are positively charged at low pH conditions and are excluded from absorption through aqueous paracellular pathway. This could be another possible explanation for the high lag times and low permeabilities of the phenylephrone esters at low pH conditions.

Conclusions

This paper reports the transport characteristics of the novel mydriatic agents and also the use of SIRC rabbit corneal cell line as an in vitro cell culture model for assessing corneal transport of drug molecules. The SIRC cell layers when grown on polycarbonate membranes exhibited high TEER and a low permeability to the hydrophilic marker, mannitol. This barrier property functionally characterizes these cell lines as "tight" ion transporting cell layers. Testosterone (a lipophilic molecule with a log *P* of 3.31), however, showed a high permeability across the SIRC cells. The results of this investigation suggest that the SIRC cell line, when grown on polycarbonate membranes, is a valid in vitro cell culture model for the study of corneal absorption and transepithelial transport of drugs. Studies on the permeability characteristics of novel mydriatic agents across the SIRC cell layers have shown that the esters of phenylephrone had higher transport rates compared to phenylephrine. The rate and extent of the transport of these compounds are influenced by the fraction of ionized and un-ionized species (which in turn depends on the pK_a of the drug and the pH of the solution) and the intrinsic partition coefficient of the drug. Studies are in progress to test the pharmacological activity and to assess the in vivo distribution and metabolism of the novel compounds in rabbits eyes.

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