

Effect of Hydroxypropyl Beta Cyclodextrin Complexation on Aqueous Solubility, Stability, and Corneal Permeation of Acyl Ester Prodrugs of Ganciclovir

Submitted: November 22, 2002; Accepted: April 21, 2003

Giridhar S. Tirucherai^{1,2} and Ashim K. Mitra¹

¹Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, 5005 Rockhill Road, Kansas City, MO 64110

²Department of Clinical Pharmacology, Quintiles Inc, 10245 Hickman Mills Drive, Kansas City, MO 64137

ABSTRACT

The purpose of the study was to investigate the effect of hydroxypropyl beta cyclodextrin (HP β CD) on aqueous solubility, stability, and in vitro corneal permeation of acyl ester prodrugs of ganciclovir (GCV). Aqueous solubility and stability of acyl ester prodrugs of Ganciclovir (GCV) were evaluated in pH 7.4 isotonic phosphate buffer solution (IPBS) in the presence and absence of HP β CD. Butyryl cholinesterase-mediated enzymatic hydrolysis of the GCV prodrugs was studied using various percentage w/v HP β CD. In vitro corneal permeation of GCV and its prodrugs (with and without 5% HP β CD) across isolated rabbit cornea was studied using side-by-side diffusion cells. HP β CD-prodrug complexation was of the A_L type with values for complexation constants ranging between 12 and 108 M⁻¹. Considerable improvement in chemical and enzymatic stability of the GCV prodrugs was observed in the presence of HP β CD. The stabilizing effect of HP β CD was found to depend on the degree of complexation and the degradation rate of prodrug within the complex. Five percent w/v HP β CD was found to enhance the corneal permeation of only the most lipophilic prodrug GCV dibutyrate (2.5-fold compared with 0% HP β CD). All other prodrugs showed little or no difference in transport in the presence of 5% w/v HP β CD. Agitation in the donor chamber largely influenced the transport kinetics of GCV dibutyrate across cornea.

Results indicate the presence of an unstirred aqueous diffusion layer at the corneal surface that restricts the transport of the highly lipophilic GCV dibutyrate prodrug. HP β CD improves corneal permeation by solubilizing the hydrophobic prodrug and delivering it across the mucin layer at the corneal surface.

KEYWORDS: hydroxypropyl beta cyclodextrin, cornea, transport, prodrugs, aqueous diffusion layer

INTRODUCTION

Ganciclovir (GCV), an acyclic guanosine analog exhibits excellent antiviral activity against herpes family of viruses such as human cytomegalo virus (HCMV), Epstein-Barr, herpes simplex virus (HSV), and varicella zoster virus (VZV). As a result of its broad spectrum of antiviral activity, GCV has been used extensively in the eye to treat anterior as well as posterior chamber infections.^{1,2} The drug exhibits a remarkable effect against HSV-1-mediated epithelial and stromal keratitis in rabbits as well as humans.^{3,4} This antiviral agent has also been shown to be very effective in treating CMV-mediated retinal infection.⁵ However, poor ocular membrane uptake due to relatively low partition coefficient severely limits the utility of GCV for corneal as well as retinal treatment. Previous reports from our laboratory have illustrated the utility of an acyl ester prodrug approach toward achieving enhanced GCV uptake across the corneal epithelium⁶ as well as the retina.⁷ The prodrugs, by virtue of their enhanced partitioning onto the respective ocular membranes, resulted in higher ocular bioavailability than the parent drug.

In the present study, a cyclodextrin-prodrug (CD-PD) complexation strategy was adopted with a view toward enhancing the aqueous solubility, stability, and corneal

Corresponding Author: Ashim K. Mitra, Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, 5005 Rockhill Road, Kansas City, MO 64110. Phone: (816) 235-1615; Fax: (816) 235-5190; Email: mitraa@umkc.edu

permeation of the acyl ester prodrugs of GCV. It was hypothesized that this strategy would allow an increase in aqueous solubility without a change in the molecular structure and the intrinsic ability of the lipophilic esters to partition onto biological membranes. Cyclodextrins are cyclic oligosaccharides with a hydrophilic outer surface and a hydrophobic central cavity. The hydrophilic exterior renders the cyclodextrin water soluble and the hydrophobic interior provides a microenvironment for relatively nonpolar drugs. In aqueous solutions, cyclodextrins can form inclusion complexes with lipophilic prodrugs by entrapping either the entire prodrug molecule or a nonpolar part of it inside the hydrophobic cavity.⁸ Such encapsulation may protect the prodrug against potential degradation by corneal membrane-bound esterases.

Several previously published reports have documented the use of cyclodextrins to improve solubility, stability, and bioavailability of ophthalmic drugs.⁹⁻¹² However, a majority of these studies have selected highly lipophilic drugs. The major aim of the present study was to investigate the complexation potential of a series of short-chain acyl ester prodrugs encompassing a wide range of lipophilicity to determine if desirable increases in solubility, stability, and permeation can be obtained for all prodrugs. In a previous article, the corneal permeation of GCV and its mono acyl ester prodrugs was reported.⁶ In the present study, the permeability of 2 diester prodrugs of GCV, namely, GCV dipropionate (GCV DP) and GCV dibutyrate (GCV DB) was determined to delineate the effect of di-substitution of the GCV side chain on corneal permeation. In addition, the effect of HP β CD complexation on permeation of GCV and its mono- and diester prodrugs was also investigated. HP β CD was selected as the model cyclodextrin in these studies because its safety over other cyclodextrins for ocular administration has been well documented.^{12,13} In vitro corneal permeation studies were carried out to delineate the effect of HP β CD complexation on drug/prodrug absorption.

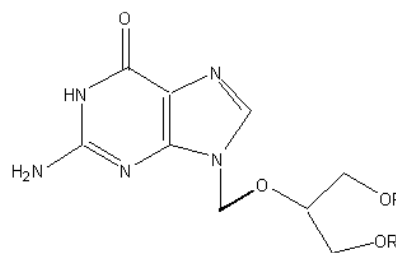
MATERIALS AND METHODS

GCV was a gift from Hoffman La Roche (Nutley, NJ). All other chemicals (buffer components) were obtained from Sigma Chemical Company (St Louis, MO). **Scheme 1** depicts the structures of GCV and the short-chain ester prodrugs employed in the present study. The regio-selective synthesis of various short-chain acyl ester prodrugs of GCV was recently reported.¹⁴

Samples were analyzed by high-performance liquid chromatography (HPLC). Methods capable of simulta-

neous analysis of the parent drug and the short chain ester prodrug were developed. The analytical methodology has been described in detail elsewhere.⁶

HP β CD (average molecular weight 1600) was purchased from Acros Chemicals (Somerville NJ). The solvents were of analytical grade and obtained from Fisher Scientific (Fairlawn, NJ). Pure butyryl cholinesterase was obtained from Sigma.



Compound	R	R'
Ganciclovir (GCV)	H	H
Monoacetate (GCV MA)	H	COCH ₃
Monopropionate (GCV MP)	H	COCH ₂ CH ₃
Monobutyrate (GCV MB)	H	CO(CH ₂) ₂ CH ₃
Monovalerate (GCV MV)	H	CO(CH ₂) ₃ CH ₃
Dipropionate (GCV DP)	COCH ₂ CH ₃	COCH ₂ CH ₃
Dibutyrate (GCV DB)	CO(CH ₂) ₂ CH ₃	CO(CH ₂) ₂ CH ₃

Scheme 1

Animals

Adult male New Zealand albino rabbits weighing between 5 and 6 lbs were obtained from Myrtle's Rabbitry (Thompson Station, TN). The investigations utilizing animals described in this report conformed to guidelines established by the Association for Research in Vision and Ophthalmology (ARVO).

Phase Solubility Studies

The complexation of GCV and its mono- and diester prodrugs was determined by using the phase solubility method of Higuchi and Connors.¹⁵ Excess amount of GCV or its prodrug was added to isotonic phosphate buffer solution (IPBS) (pH 7.4) containing increasing percentage w/v (1%, 2%, 5%, 8%, and 10%) of HP β CD. The suspensions were shaken at 34°C for 24 hours. After equilibration, the suspensions were filtered using 0.45- μ m membrane filters, and solubility was determined by HPLC analysis. Intrinsic solubility of GCV and its prodrugs was also determined in IPBS devoid of cyclodextrin (0% CD).

Chemical Stability Studies

Stability of the prodrugs in pH 7.4 IPBS at 34°C in the absence and presence of HP β CD was determined. Aliquots (10 mL) of isotonic phosphate buffer were placed in screw-capped vials and allowed to equilibrate at 34°C. Prodrug stock solution (50 μ L) in dimethyl sulfoxide (DMSO) was subsequently added to the buffer to yield a concentration of 20 μ M. The vials were placed in a constant shaker bath set at 34°C and 60 rpm. Samples (100 μ L) were collected at appropriate time intervals for up to 72 hours and stored at -80°C until further analysis. Linear regression of the log concentration versus time profiles yielded the pseudo first order rate constants of degradation. Degradation studies were also carried out using 5% HP β CD in IPBS to study the effect of cyclodextrin on chemical stability of the prodrugs.

Enzymatic Hydrolysis Studies

Hydrolysis studies were carried out in the presence and absence of HP β CD to determine if cyclodextrin complexation can protect the GCV prodrugs against enzymatic degradation by membrane-bound enzymes of the cornea (such as acetyl and butyryl cholinesterases).

Butyryl cholinesterase-mediated enzymatic degradation of GCV MP and GCV MV was studied in the presence of increasing concentrations of HP β CD (0%, 5%, 10%, and 20% w/v) at 34°C. Fifty μ L of the prodrug solution in DMSO was spiked into 10 mL of pH 7.4 IPBS containing 0%, 5%, 10%, or 20% HP β CD. Final prodrug concentration was 40 μ M. The solution was pre-equilibrated for 15 minutes at 34°C. Just prior to the addition of enzyme, the mixture was vortexed for 5 seconds and a 100- μ L sample was taken as the zero time point. Subsequently, 100 μ L stock solution of the enzyme in IPBS was added to the reaction mixture to yield an enzyme concentration of 2 units/mL. Aliquots (100 μ L) were withdrawn and 100 μ L of chilled methanol was added to stop the reaction. Samples were withdrawn every 15 minutes for the first hour and every 30 minutes for the next 2 hours, and the samples were subsequently stored at -80°C until further analysis was performed. Studies were performed in triplicate.

In Vitro Permeability Studies

Corneal membrane permeation studies were carried out using excised corneas of male New Zealand albino rabbits. Animals were euthanized by an overdose of pentobarbital administered through the marginal ear

vein. The cornea was excised according to a previously reported procedure.¹⁶ Immediately following excision, the cornea was washed with ice-cold IPBS pH 7.4 and mounted on side-by-side diffusion chambers with the epithelial side facing the donor compartment. Temperature was maintained at 34°C (temperature of rabbit cornea) by circulating water through the jacketed chambers of the diffusion apparatus. Both half-cells were placed on automated drive consoles in order to continuously stir the contents of the half-cells. Experiments were conducted to delineate the effect of the following variables on corneal permeation:

1. Diester modification

2. HP β CD

a. Membrane Alteration: Permeation of ¹⁴C mannitol (1.0 μ Ci/mL), a paracellular marker in the presence of various concentrations of HP β CD (0%, 5%, 10%, and 20% w/v), was carried out to determine an optimal concentration of HP β CD that can be used in transport studies without causing disruption of the tight junctions in the corneal epithelium.

b. Drug/prodrug transport enhancement: Saturated solutions of GCV and its mono- and diester prodrugs, pre-equilibrated for 24 hours at 34°C in the presence and absence of HP β CD, were added to the donor side to determine the effect of complexation on drug and prodrug permeation.

3. Mixing conditions

Corneal permeation of selected prodrugs (GCV MV and GCV DB) was determined in the presence and absence of stirring in the donor chamber to investigate the presence and role of an unstirred aqueous diffusion layer at the corneal surface on permeation of hydrophobic compounds.

The compound of interest (in IPBS) was added on the epithelial side of the cornea (donor chamber) at concentrations of saturation solubility (**Table 1**). In the other half chamber (receiver chamber), 3.2 mL of pH 7.4 IPBS was added. The receiver chamber volume of IPBS added was slightly more than that of the donor chamber in order to maintain the natural curvature of the cornea throughout the experiment. The contents in both chambers (or only the receptor chamber in the case of stagnant diffusion layer studies) were stirred continuously by using magnetic stir bars. Total duration of a transport study was 3 hours. Samples of 200 μ L were removed from the receiver chamber every 15 minutes for the first hour and every 30 minutes for the next 2 hours. Samples were immediately replaced with an equal volume of pH 7.4 IPBS to ensure sink

Table 1. Solubility, Octanol/Buffer Partition Coefficient, and Complexation Constants for GCV Prodrugs*

Compound	Log P _{O/B} [19]	Solubility (mM)			K _{1:1} (M ⁻¹)
		0% CD	5% CD	10% CD	
GCV MA	-1.08	12.22	16.91	20.71	12.02
GCV MP	-0.92	10.51	15.61	21.02	18.71
GCV MB	-0.30	5.60	9.82	13.31	24.42
GCV MV	-0.07	3.71	8.91	14.03	50.03
GCV DP	-0.23	6.01	13.37	18.07	39.71
GCV DB	0.59	0.42	1.92	3.03	106.71

*GCV indicates ganciclovir; P_{O/B}, octanol-pH 7.4 buffer partition coefficient; K_{1:1}, complexation constant; CD, cyclodextrin; MA, monoacetate; MP, monopropionate; MB, monobutyrate; MV, monovalerate; DP, dipropionate; DB, dibutyrate.

conditions. The samples were analyzed for intact prodrug as well as regenerated GCV by HPLC. All experiments were conducted at least in triplicate.

Determination of Transport Parameters

The cumulative amount of total GCV (sum of intact prodrug and regenerated GCV in molar quantities) in the receptor phase was plotted as a function of time to determine prodrug permeability. Linear regression of the total GCV amounts yielded the rate of transport of the prodrug across the cornea (dM/dt). The rate divided by the area available for diffusion (A) generates the steady state flux as shown in Equation 1 below:

$$\text{Flux} = (\text{dM/dt})/A \quad (1)$$

Corneal permeabilities were calculated by dividing the steady state flux by the donor concentration (C_d) of the prodrug according to Equation 2.

$$\text{Permeability} = \text{Flux}/C_d \quad (2)$$

RESULTS

Phase Solubility Studies

Phase solubility studies of various GCV prodrugs in aqueous HPβCD solutions were carried out at pH 7.4 and 34°C. Solubility of the prodrugs increased linearly with increasing concentrations of HPβCD. Extent of solubility enhancement with 10% HPβCD ranged from 1.7-fold for GCV MA (the smallest chain-length homolog) to 7.3-fold for GCV DB (the highest chain-length homolog). Solubility enhancement of the diesters was significantly higher than the respective mono-

esters. GCV did not show any improvement in solubility indicating that it does not complex with HPβCD.

Phase solubility diagrams of GCV and its prodrugs were of A_L type indicating the formation of 1:1 prodrug/HPβCD complexes within the concentration range of cyclodextrin used.¹⁵ Apparent stability constants for 1:1 complexes (K_{1:1}) were calculated using Equation 3.

$$K_{1:1} = \text{slope}/[S_0*(1-\text{slope})] \quad (3)$$

S₀ is the intrinsic solubility of the prodrug (ie, in 0% HPβCD).

Table 1 summarizes the prodrug/HPβCD complexation constants and aqueous solubility of GCV prodrugs in the presence of 0%, 5%, and 10% HPβCD.

Chemical Stability Studies

Table 2 lists the degradation rate constants of the prodrugs at 34°C in pH 7.4 IPBS. There was no significant change in the rate of GCV MA degradation as a result of complexation. However, all the other prodrugs showed significant reduction in chemical degradation with 5% HPβCD. **Table 2** also reveals that higher increase in stability is achieved with the prodrugs having higher complexation constants with HPβCD.

Enzymatic Hydrolysis Studies

Degradation of the prodrugs by butyryl cholinesterase followed apparent first order kinetics. **Table 3** depicts the values of the observed first order degradation rate constants (k_{obs}) of the prodrug at 0%, 5%, 10%, and 20% HPβCD. GCV MV exhibited greater stability than GCV MP at all concentrations of HPβCD. It is evident from **Table 3** that resistance to enzymatic degradation

Table 2. Chemical Hydrolysis Rate Constants of GCV Prodrugs in the Presence and Absence of 5% HPβCD*

Compound	K _{1:1} (M ⁻¹)	Rate Constant of Chemical Hydrolysis (k [†] × 10 ⁴ h ⁻¹)	
		0% HPβCD	5% HPβCD
GCV MA	12.0	16.64 ± 0.32	15.84 ± 0.74 [†]
GCV MP	18.7	18.69 ± 1.38	13.12 ± 1.91
GCV MB	24.4	10.27 ± 1.43	6.73 ± 0.92
GCV MV	50.0	18.8 ± 2.45	9.78 ± 1.11
GCV DP	39.7	32.43 ± 4.31	21.09 ± 3.23
GCV DB	106.7	43.61 ± 4.65	13.21 ± 2.14

*Studies were carried out in pH 7.4 isotonic phosphate buffer solution (IPBS) at 34°C. Abbreviations are explained in the first footnote to Table 1. Values are mean ± SD (n = 3).

[†]indicates *P* > 0.05 in comparison to 0% HPβCD.

Table 3. Enzymatic Hydrolysis Rate Constants of GCV Mono-esters in the Presence of Increasing Concentrations of HPβCD*

Prodrug	Rate Constant of Enzymatic Hydrolysis (k _{obs} × 10 ³) min ⁻¹			
	0% HPβCD	5% HPβCD	10% HPβCD	20% HPβCD
GCV MP	2.32 ± 0.11	1.80 ± 0.07	1.61 ± 0.09	1.29 ± 0.19
GCV MV	7.31 ± 0.04	3.70 ± 0.14	2.81 ± 0.17	2.10 ± 0.09

*GCV indicates ganciclovir; HPβCD, hydroxypropyl beta cyclodextrin; k_{obs}, observed hydrolytic rate constants; MP, monopropionate; and MV, monovalerate. Values are mean ± SD (n = 3).

of the prodrugs was greater with increasing concentration of HPβCD.

In Vitro Permeation Studies

Corneal Permeation of GCV Diesters

Corneal permeation of 2 diester prodrugs was studied. **Figures 1** and **2** illustrate permeation profiles of the dipropionate and the dibutyrate esters of GCV. The accumulation of the intact diester, the hydrolyzed monoester, and the hydrolyzed parent drug (GCV) appears to be linear with time for both prodrugs. Simultaneous hydrolysis during corneal transit of the intact diester caused the formation of the monoester and subsequently the parent drug GCV. The hydrolyzed monoesters were the predominant permeating species. Permeabilities of both diesters were determined from the linear steady state portion of the total GCV permeated versus time profile as described under the “methods” section. The permeability of the diesters in relation to the monoesters is listed in **Table 4**. Permeability of GCV was enhanced by ester prodrug modification, and

the diesters permeate the cornea at a faster rate than the monoesters.

Permeation of Marker Compound

Corneal transport profiles of ¹⁴C mannitol in the presence of increasing HPβCD concentrations are shown in **Figure 3**. Although 5% HPβCD did not show any significant difference from the control, both time-dependent and concentration-dependent loss of linearity in the profile were observed with the use of 10% and 20% HPβCD. Such drastic deviation from linearity of the profile indicates that the use of HPβCD beyond 5% w/v probably leads to disruption of tight junctions of the corneal epithelium. Based on these results, 5% HPβCD was selected for further transport experiments.

Effect of HPβCD Complexation on Permeation

Corneal transport of GCV and its prodrugs was compared in the absence and presence of HPβCD (5%). Although HPβCD increased the solubility of all the

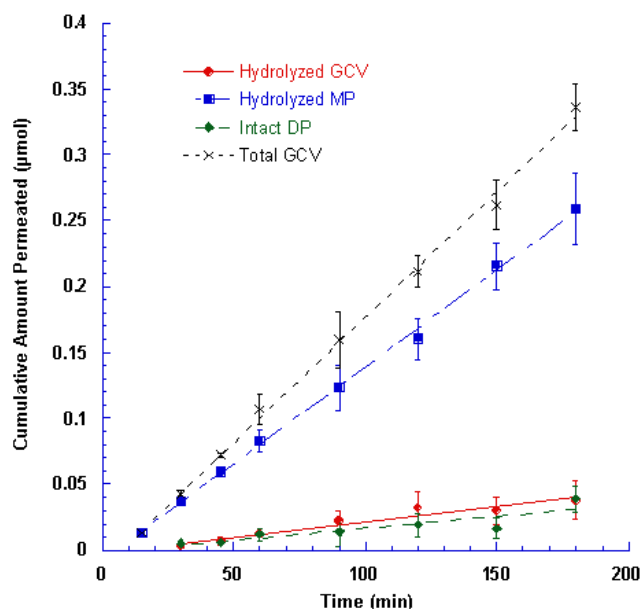


Figure 1. In vitro permeation of GCV DP prodrug across excised rabbit cornea. Values are mean \pm SD (n = 4 to 6).

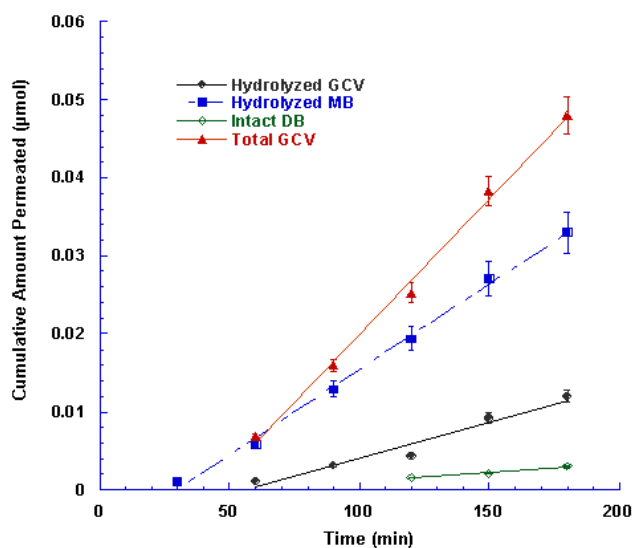


Figure 2. In vitro permeation of GCV DB prodrug across excised rabbit cornea. Values are Mean \pm SD (n = 4 to 6).

ester prodrugs, it did not produce a statistically significant increase in the permeation of total GCV except in case of the dibutyrate ester. In case of GCV DB, the lag phase was reduced, and the amounts of total GCV permeated was increased approximately 2.5-fold with 5% HP β CD compared with 0% HP β CD (**Figure 4**).

Table 4. Relative Corneal Permeabilities of GCV Mono- and Diesters*

Compound	Corneal Permeability $\times 10^6$ (cm/sec)
GCV	3.82 \pm 0.19
GCV MP	5.70 \pm 0.11
GCV DP	8.32 \pm .132
GCV MB	7.66 \pm 0.37
GCV DB	22.17 \pm 1.12

*Abbreviations are explained in the first footnote to Table 1. Values are mean \pm SD (n = 4 to 6).

Effect of Donor Phase Agitation on Prodrug Permeation

To investigate the mechanism of GCV permeation enhancement by GCV DB prodrug, transport studies were conducted in the presence and absence of stirring and with and without HP β CD in the donor chamber of the diffusion cells. A remarkable decrease in the transport rate (up to 2.5-fold) was observed in the absence of stirring when no HP β CD was present in the donor solution (**Figure 5**). Quantitation of the diester was not possible in the receptor compartment. The lag phase observed was greater when static conditions were maintained. However, similar studies with GCV DB in the presence of 5% HP β CD showed only a slight difference ($P < 0.05$) in transport when static conditions were maintained in the donor chamber (**Figure 6**).

Corneal permeation of GCV MV, the most lipophilic monoester was also evaluated in the presence and absence of stirring and with and without HP β CD. GCV MV (0% CD) showed a slight but significant decrease in transport in the absence of stirring in the donor chamber (**Figure 7**). However, with the inclusion of 5% HP β CD in solution, this small difference in transport rate was almost eliminated (**Figure 8**). These results indicate that an unstirred water layer capable of restricting the transport of highly hydrophobic molecules is probably present on the corneal interface. The results also indicate that HP β CD minimizes the barrier property of this aqueous boundary layer by rendering the prodrugs more hydrophilic through inclusion complex formation.

DISCUSSION

Phase Solubility Studies

All prodrugs exhibited higher solubility as a result of complexation with HP β CD. The extent of increase in solubility was consistently greater as the chain length

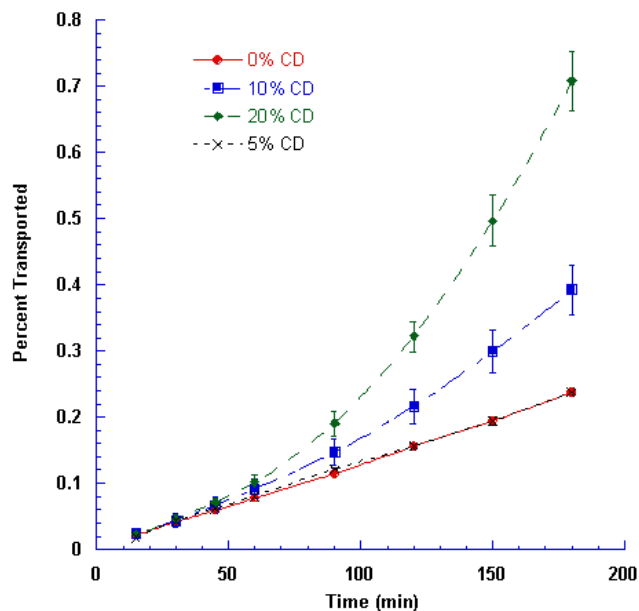


Figure 3. Effect of percentage HP β CD on corneal permeation of ^{14}C mannitol. Values are mean \pm SD (n = 4).

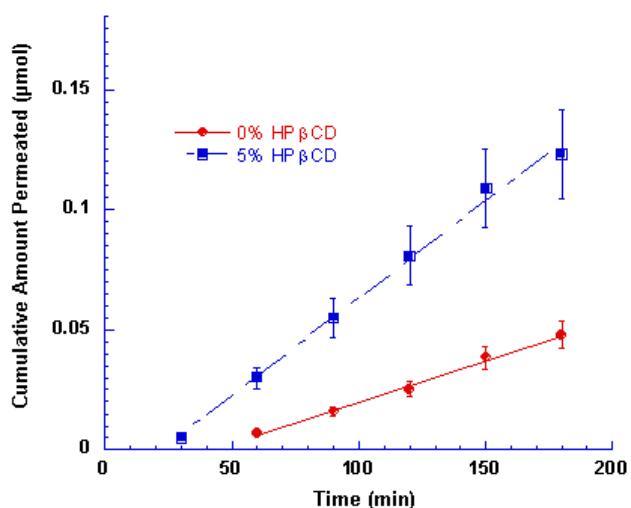


Figure 4. Effect of HP β CD on corneal permeation of GCV DB. Values are mean \pm SD (n = 4 to 6).

was ascended indicating an interaction or inclusion of the nonpolar side chain in the hydrophobic HP β CD cavity. A direct relationship between prodrug lipophilicity and complexation constant has also been es-

tablished (Table 1). A similar relationship between lipophilicity and inclusion complexation has been previously reported.^{17,18} However, the values obtained for the stability constants of the prodrugs/HP β CD complexes were relatively low (12 M^{-1} to 108 M^{-1}).

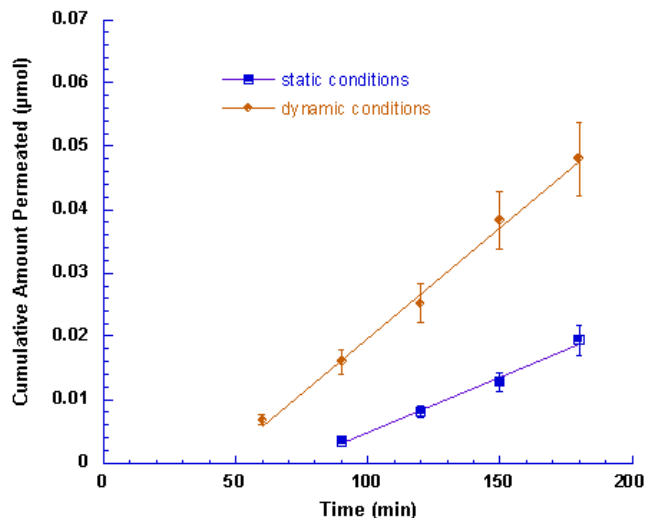


Figure 5. Effect of stirring on transport kinetics of GCV DB (0% HP β CD). Values are mean \pm SD (n = 4 to 6).

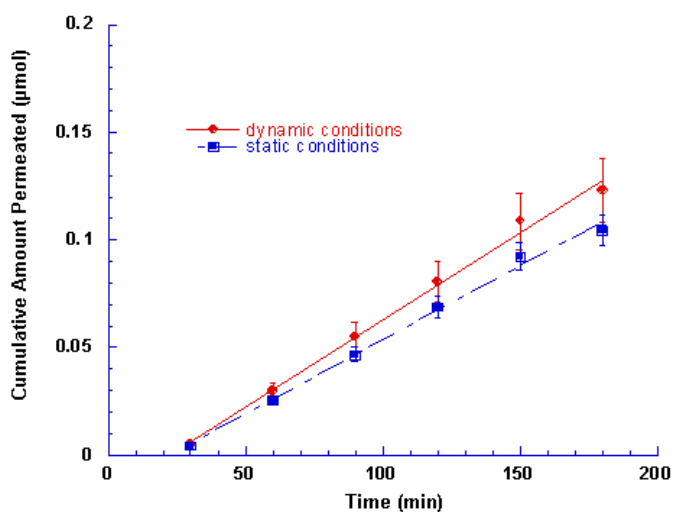


Figure 6. Effect of stirring on transport kinetics of GCV DB (5% HP β CD). Values are mean \pm SD (n = 4 to 6).

Stability Studies

The most common dosage form for topical ocular administration is the aqueous solution as it is a convenient form of delivery, is relatively inexpensive, and does not impair vision. However, in aqueous solution,

most drugs are prone to chemical degradation. It was shown earlier that GCV prodrugs exhibited maximal stability at pH 4.0 and in generating a better stability under acidic than alkaline pH.¹⁹ However, decreasing the pH of a solution to achieve stability may lead to an enhancement in ocular irritation. To avoid this, pH 7.4 IPBS was used for all permeation studies.

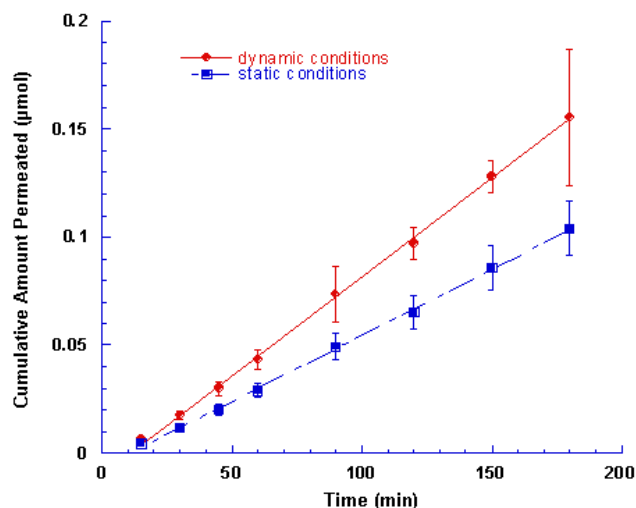


Figure 7. Effect of stirring on transport kinetics of GCV MV (0% HPβCD). Values are mean ± SD (n = 4 to 6).

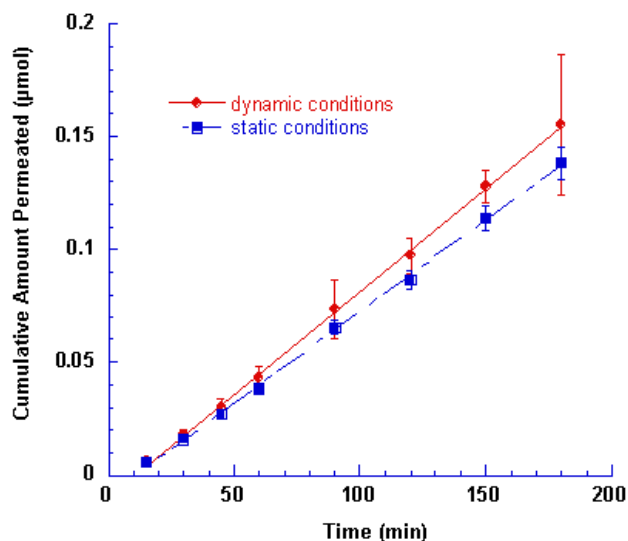
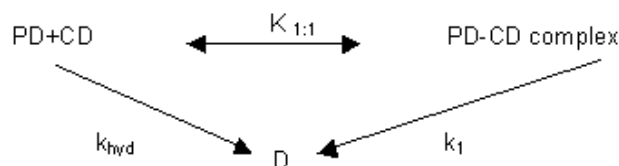


Figure 8. Effect of stirring on transport kinetics of GCV MV (5% HPβCD). Values are mean ± SD (n = 4 to 6).

One of the most common pharmaceutical applications of cyclodextrins is to enhance drug stability in aqueous solutions.^{20,21} Inclusion complex formation may be regarded as an encapsulation of the prodrug molecule or at least a labile ester segment of the molecule. Entrapment can protect the prodrug against attack by various reactive species. Increased stability of the prodrugs in aqueous HPβCD solutions indicated that the ester moiety is at least partially enclosed in the cyclodextrin cavity. It is possible that the ester linkage is located in the apolar region of the cyclodextrin molecule. As such, the linkage is sterically stabilized from the hydroxyl groups on the surface of the cyclodextrin molecule, which can otherwise mediate a nucleophilic attack on the carbonyl function of the prodrug molecule causing hydrolysis.

The stabilizing effect of CDs depends on the degree of complexation and on the degradation rate of prodrug within the complex. The degradation kinetics of a prodrug forming inclusion complex with CD is shown in Scheme 2.



Scheme 2

The prodrugs may interact with cyclodextrin to generate inclusion complexes (PD-CD). The free as well as the complexed prodrug can undergo hydrolysis to generate active drug (D). The first order hydrolysis rate constant for the free prodrug is k_{hyd} , and k_1 is that of the complexed prodrug. Prodrug stabilization may be enhanced by a higher complexation constant ($K_{1:1}$) and/or a lower hydrolytic rate constant (k_1).

Prodrug stability against enzymatic degradation using various concentrations of HPβCD was studied. The observed hydrolytic rate constants (k_{obs}) significantly decreased with increasing percentage HPβCD as shown in Table 3. Therefore k_{obs} is the weighted average of the 2 hydrolytic rate constants of the free prodrug (k_{hyd}) and the complexed prodrug (k_1). It is possible to estimate the values of the rate of degradation of the complexed prodrug by using a Lineweaver Burke type of equation (Equation 4).²⁰

$$1/(k_{\text{hyd}} - k_{\text{obs}}) = 1/K_{1:1}(k_{\text{hyd}} - k_1) * 1/[CD] + 1/k_{\text{hyd}} - k_1 \quad (4)$$

where [CD] is the concentration of total HP β CD in the reaction medium. A plot of $1/(k_{\text{hyd}} - k_{\text{obs}})$ versus $1/[\text{CD}]$ will generate a straight line (if the assumption of a 1:1 stoichiometry is correct) with a y intercept equal to $1/(k_{\text{hyd}} - k_1)$ and a slope equal to $1/K_{1:1}(k_{\text{hyd}} - k_1)$. This relationship not only allows for the prediction of the rate constant for hydrolysis of the complexed prodrug but also provides an independent estimate of the value of the complexation constant, originally estimated using phase solubility analysis.

Figure 9 shows a linear relationship between $1/(k_{\text{hyd}} - k_{\text{obs}})$ and $1/[\text{CD}]$. Estimates of the values of k_{hyd} , k_1 , and $K_{1:1}$ are summarized in **Table 5**. Hydrolytic rate constants for the complexed prodrug (k_1) were 2.5- and 6-fold less than that of the free drug (k_{hyd}) for GCV MP and GCV MV, respectively, indicating that the complexed prodrug hydrolyzed at a much slower rate compared with the uncomplexed prodrug. The degree of stabilization of the prodrug not only depends on the rate of hydrolysis of the prodrug in the complex but also on the fraction of the prodrug that resides within the complex (which in turn depends on the $K_{1:1}$). GCV MV had a higher complexation constant with HP β CD, which increased the fraction of complexed prodrug in the solution, thereby resulting in greater stabilization than GCV MP.

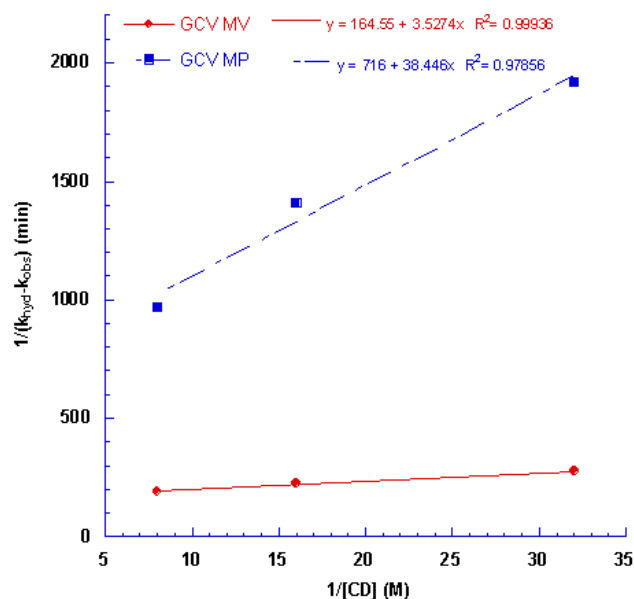


Figure 9. Estimation of k_1 and $K_{1:1}$ from enzymatic hydrolysis.

In Vitro Corneal Permeation Studies

Permeation of GCV Diesters

GCV structure contains 2 primary hydroxyl groups, either or both of which can be esterified to form mono- or diesters. The corneal permeation of GCV and its monoester prodrugs was reported previously.⁶ In this study, the potential advantage of di-substitution over mono-substitution was investigated. Two diester prodrugs, GCV dipropionate (GCV DP) and GCV dibutyrate (GCV DB), were selected as model compounds. GCV DB is significantly less soluble and more lipophilic than GCV DP (**Table 1**). As a result of its poor solubility, GCV DB permeated in low quantities. A pronounced lag phase in the transport profile was observed (**Figure 2**). The major permeating species was the hydrolyzed monoester (GCV MB). Ocular bioreversibility studies using corneal homogenate have established that the diester to monoester conversion rate is fairly rapid for GCV DB.¹⁹ Accordingly, only the last 3 time points showed any measurable concentrations of the intact prodrug in the receptor medium. However, in the case of GCV DP, the rate of conversion of the diester to the monoester is slower, therefore considerable permeation of the intact diester was observed (**Figure 1**).

Permeability values of GCV DP and GCV DB and the respective monoesters (ie, GCV MP and GCV MB) are summarized in **Table 4**. Higher permeability values were obtained for the diesters indicating that di-substitution imparts increased lipophilicity, which leads to increased permeability compared with the mono-substituted GCV.

However, the permeability value of GCV DB represents a paradox in corneal transport. Although higher permeability value was achieved with the dibutyrate ester, its steady state flux from a saturated solution across the cornea was much lower than any of the monoesters. Flux is the therapeutically relevant transport parameter because it determines the maximum steady state concentrations of drug that can be achieved following transmembrane transport. A high permeability value of GCV DB was obtained as a result of the extremely low concentration of GCV DB (saturation solubility) in the donor chamber (since permeability = flux/donor concentration). However, greater permeability does not indicate a greater total amount transported across the cornea because a decrease in solubility may override permeability enhancement.

The need for a balance between lipophilicity and solubility in ophthalmic formulations has been emphasized previously.²² Poor solubility presents a formulation

Table 5. Comparison of Free Versus Complexed Prodrug Hydrolysis*

Prodrug	$k_{\text{hyd}} \times 10^3 \text{ min}^{-1}$	$K_1 \times 10^3 \text{ min}^{-1}$	$K_{1:1} (\text{M}^{-1})$
GCV MP	2.32	0.90	18.62
GCV MV	7.31	1.21	46.5

* k_{hyd} indicates first order hydrolysis rate constant for the free prodrug; MP, monopropionate; MV, monovalerate. Studies were carried out in pH 7.4 IPBS at 34°C.

challenge because the amount of drug that can be formulated in solution is relatively low. In order to overcome this formulation constraint, we adopted a prodrug complexation approach. Prodrug-HP β CD inclusion complexation strategy allows an increase in aqueous solubility without a change in the molecular structure or the intrinsic ability of the lipophilic esters to partition onto lipoidal corneal epithelium.

Effect of HP β CD on Corneal Permeation of GCV and Its Prodrugs

Cyclodextrins have been used in drug development to achieve increased solubility, stability, and bioavailability of therapeutic agents. Incorporation of cyclodextrins in ophthalmic formulations has been shown to be beneficial in applications ranging from enhanced precorneal retention of drug to decreased ocular irritation.^{23,24} Solubility enhancement studies with HP β CD for GCV prodrugs showed substantial increase in solubility for all esters. This investigation attempted to answer whether cyclodextrin complexation of a series of increasingly lipophilic ester prodrugs of GCV would confer increased corneal permeation as a result of enhancement in solubility and stability.

It was first necessary to establish the safety of the chosen cyclodextrin for ophthalmic use. Selection of HP β CD was based on the fact that this substituted cyclodextrin possesses high aqueous solubility and complexation ability. Several studies have demonstrated the safety as well as ocular tolerance of HP β CD.^{12,13} However, these studies were carried out under normal physiological conditions. Present studies using excised rabbit cornea revealed that transport of ¹⁴C mannitol, an established paracellular marker, increased in a time-dependent manner at concentrations greater than 10% HP β CD. It is possible that cyclodextrins are better tolerated under normal physiological conditions, where factors such as blink rate, tear flow, and drainage may serve to counteract the potentially damaging effects of cyclodextrins. Present studies indi-

cate that damage to corneal epithelial tight junctions may occur at concentrations at and beyond 10% HP β CD as a result of the cornea being in contact with the cyclodextrin solution for 3 hours. As a result, 5% HP β CD was selected as the cut-off cyclodextrin concentration for in vitro prodrug permeation studies. Although the solubility and stability of all prodrugs was increased at 5% HP β CD, only the dibutyrate ester prodrug modification resulted in a substantial increase in GCV corneal permeation. Cyclodextrin complexation leads to increased solubility because the apolar ester substituent is included within the cavity forming a PD-CD complex. However, the free fraction (ie, the uncomplexed prodrug) is the only form capable of diffusion across biological membranes. Bulky cyclodextrins cannot pass through the intercellular spaces because the molecules are larger (outer diameter of β CD is 15 Å) than the size of the tight junctional space (4-5 Å).^{25,26} Thus, it was not unexpected that the transport of all but one of the prodrugs across the cornea was unaltered when used in the presence of HP β CD. However, a remarkable increase in the transport rate (2.56-fold) of GCV DB was observed (**Figure 4**). The mechanism of HP β CD-mediated corneal permeation of GCV DB was subsequently investigated.

Mechanism of HP β CD-Mediated Corneal Permeation of GCV DB

At least 3 possible mechanisms exist by which CDs can produce enhancement in GCV DB transport. These include (1) extraction of membrane lipids, (2) increased rate of dissolution, and (3) enhanced stability of the complex. First, studies with ¹⁴C mannitol proved that the use of 5% HP β CD is safe in transport experiments. Second, the complexed prodrug solutions were pre-equilibrated for 24 hours prior to transport studies. This time was sufficient for all prodrugs to attain equilibrium solubility.¹⁹ Hence, increased dissolution rate could not explain the increase in GCV DB transport. The third and final possibility is the stabilization by

complexation (<10% hydrolysis) of GCV DB during the pre-equilibration phase and during the transport experiment. However, the magnitude of improvement in stability alone may not explain more than 100% increase in transport of GCV DB with 5% HP β CD. Further, lack of permeation enhancement of all the esters versus the dibutyrate ester showed that a different mechanism may be involved in HP β CD-mediated transport enhancement of GCV DB.

Recent reports have suggested that cyclodextrins can play a role in carrying drug molecules across aqueous diffusion layers.^{17,27,28} An aqueous mucin layer has been known to exist on the corneal and conjunctival epithelia.²⁹ Mucus in the eye is primarily produced by the conjunctival goblet cells. This mucus layer consists of dissolved inorganic salts, glucose, and urea. It is possible that the delivery of prodrugs through the aqueous mucin layer is diffusion controlled, but prodrug delivery across the cornea is membrane controlled. To investigate whether the mucin layer present on the corneal surface can affect the diffusion of hydrophobic molecules, prodrug permeation studies were carried out under dynamic and static conditions.

Stirring rates can influence transport of hydrophobic compounds significantly.³⁰ Further, transport of increasingly lipophilic compounds is affected to a larger extent by the aqueous diffusion layer.³¹ Accordingly, the effect of stirring rates on the transport of GCV MV and GCV DB was examined. GCV MV was selected because it possesses adequate water solubility. GCV DB was selected because it is highly lipophilic and also exhibits poor solubility.

Results from the corneal permeation studies demonstrated that while the total transport of GCV MV in the absence of stirring was slightly reduced ($P < 0.05$), the transport of GCV DB was diminished drastically (**Figures 5 and 7**). Similar studies with GCV DB in the presence of cyclodextrin however did not reveal such a drastic change in the absence of stirring (**Figures 6 and 8**). These results clearly indicate that the aqueous layer on the corneal epithelial surface indeed limits the transport of the highly nonpolar GCV DB. Our results also indicated that resistance to transport of drugs can be minimized by the use of HP β CD.

Cyclodextrins appear to act as true carriers by solubilizing the hydrophobic prodrug and delivering them across the mucin layer to the surface of the cornea such that the prodrug may partition onto the cornea. The relatively hydrophilic cyclodextrin molecules have a low affinity for the lipophilic membrane and remain in the aqueous donor solution. Cyclodextrin molecules may release the prodrug following diffusion layer

transport via a collision-mediated dissociation process or by simple displacement of the prodrug molecules by epithelial lipids, which are known to form strong inclusion complexes with cyclodextrin.³²

CONCLUSION

Desirable increases in solubility and stability of GCV prodrugs can be achieved via cyclodextrin complexation. However, such an approach appears to be beneficial in enhancing the transport of only highly lipophilic prodrugs. The mechanism of transport enhancement appears to involve modulation of the aqueous diffusion layer on the corneal surface. Increase in chemical stability of the prodrugs indicates that the complexation approach may be useful in improving the shelf life of prodrug-containing ophthalmic formulations. Stability of the complexed prodrug against enzymatic hydrolysis further indicates that complexes of GCV prodrugs may be useful for sustained intravitreal administration, where a slow hydrolysis of the complexed prodrug may generate sustained GCV levels in the retina.

ACKNOWLEDGEMENTS

This study was supported by grants 2 RO1 EY 09171-09 and 2 RO1 EY 10659-08 from the National Institutes of Health, Bethesda, MD.

REFERENCES

1. Snoeck R, Schols D, Andrei G, Neyts J, De Clercq E. Antiviral activity of anti-cytomegalovirus agents (HPMPC, HPMPA) assessed by a flow cytometric method and DNA hybridization technique. *Antiviral Res.* 1991;16:1-9.
2. Smee DF, Martin JC, Verheyden JP, Matthews TR. Anti-herpesvirus activity of the acyclic nucleoside 9-(1,3-dihydroxy-2-propoxymethyl)guanine. *Antimicrob Agents Chemother.* 1983;23:676-682.
3. Naito T, Nitta K, Kinouchi Y, Shiota H, Mimura Y. Effects of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) eye drops and cyclosporine eye drops in the treatment of herpetic stromal keratitis in rabbits. *Curr Eye Res.* 1991;10(suppl):201-203.
4. Hoh HB, Hurley C, Claoue C, Viswalingham M, Easty DL, Goldschmidt P, Collum LM. Randomised trial of ganciclovir and acyclovir in the treatment of herpes simplex dendritic keratitis: a multicentre study. *Br J Ophthalmol.* 1996;80:140-143.
5. Spector SA, McKinley GF, Lalezari JP, Samo T, Andruczk R, Follansbee S, Sparti PD, Havlir DV, Simpson G, Buhles W, Wong R, Stempien M. Oral ganciclovir for the prevention of cytomegalovirus disease in persons with AIDS. Roche Cooperative Oral Ganciclovir Study Group. *N Engl J Med.* 1996;334:1491-1497.

6. Tirucherai GS, Dias C, Mitra AK. Corneal Permeation of Ganciclovir: Mechanism of Ganciclovir Permeation Enhancement by Acyl Ester Prodrug Design. *J Ocul Pharmacol Ther.* 2002;18:535-548
7. Macha S, Mitra AK. Ocular disposition of ganciclovir and its monoester prodrugs following intravitreal administration using microdialysis. *Drug Metab Dispos.* 2002;30:670-675.
8. Yaksh TL, Jang JD, Nishiuchi Y, Braun KP, Ro SG, Goodman M. The utility of 2-hydroxypropyl-beta-cyclodextrin as a vehicle for the intracerebral and intrathecal administration of drugs. *Life Sci.* 1991;48:623-633.
9. Usayapant A, Karara AH, Narurkar MM. Effect of 2-hydroxypropyl-beta-cyclodextrin on the ocular absorption of dexamethasone and dexamethasone acetate. *Pharm Res.* 1991;8:1495-1499.
10. Loftsson T, Frithriksdottir H, Thorisdottir S, Stefansson E, Sigurthardottir AM, Guthmundsson O, Sigthorsson T. 2-hydroxypropyl-beta-cyclodextrin in topical carbonic anhydrase inhibitor formulations. *Eur J Pharm Sci.* 1994;1:175-180.
11. Jarho P, Urtti A, Jarvinen K, Pate DW, Jarvinen T. Hydroxypropyl-beta-cyclodextrin increases aqueous solubility and stability of anandamide. *Life Sci.* 1996;58: PL 181-185.
12. Loftsson T, Jarvinen T. Cyclodextrins in ophthalmic drug delivery. *Adv Drug Deliv Rev.* 1999;36:59-79.
13. Loftsson T, Stefansson E, Kristensson JK, Fridriksdottir, H, Sverrisson T, Gudmundsdottir G, Thorisdottir S. Topically effective acetazolamide drop solution in man. *Pharm Sci.* 1996;6:277-279.
14. Gao H, Mitra AK. Regioselective synthesis of various prodrugs of ganciclovir. *Tetrahedron Lett.* 2000;41:1131-1136.
15. Higuchi T, Connors KA. "Phase-Solubility Techniques" In *Advances in Analytical Chemistry and Instrumentation.* New York: Reilly CN; 1965:117-212
16. Tak RV, Pal D, Gao H, Dey S, Mitra AK. Transport of acyclovir ester prodrugs through rabbit cornea and SIRC-rabbit corneal epithelial cell line. *J Pharm Sci.* 2001;90:1505-1515.
17. Cho MJ, Chen FJ, Huczek DL. Effects of inclusion complexation on the transepithelial transport of a lipophilic substance in vitro. *Pharm Res.* 1995;12:560-564.
18. Albers E, Muller BW. Complexation of steroid hormones with cyclodextrin derivatives: substituent effects of the guest molecule on solubility and stability in aqueous solution. *J Pharm Sci.* 1992;81:756-761.
19. Dias CS, Anand BS, Mitra AK. Effect of mono- and diacylation on the ocular disposition of ganciclovir: physicochemical properties, ocular bioversion, and antiviral activity of short chain ester prodrugs. *J Pharm Sci.* 2002;91:660-668.
20. Loftsson T, Brewster ME. Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. *J Pharm Sci.* 1996;85:1017-1025.
21. Mielcarek J. Photochemical stability of the inclusion complexes formed by modified 1,4-dihydropyridine derivatives with beta-cyclodextrin. *J Pharm Biomed Anal.* 1997;15: 681-686.
22. Narurkar MM, Mitra AK. Prodrugs of 5-iodo-2'-deoxyuridine for enhanced ocular transport. *Pharm Res.* 1989;6:887-891.
23. Jarho P, Jarvinen K, Urtti A, Stella VJ, Jarvinen T. Modified beta-cyclodextrin (SBE7-beta-CyD) with viscous vehicle improves the ocular delivery and tolerability of pilocarpine prodrug in rabbits. *J Pharm Pharmacol.* 1996;48:263-269.
24. Suhonen P, Jarvinen T, Lehmuusaari K, Reunamaki T, Urtti A. Ocular absorption and irritation of pilocarpine prodrug is modified with buffer, polymer, and cyclodextrin in the eyedrop. *Pharm Res.* 1995;12:529-533.
25. Pauletti GM, Gangwar S, Wang B, Borchardt RT. Esterase-sensitive cyclic prodrugs of peptides: evaluation of a phenylpropionic acid moiety in a model hexapeptide. *Pharm Res.* 1997;14:11-17.
26. Bodor NS, Huang MJ, Watts JD. Theoretical studies on the structures of natural and alkylated cyclodextrins. *J Pharm Sci.* 1995;84:330-336.
27. Masson M, Loftsson T, Masson G, Stefansson E. Cyclodextrins as permeation enhancers: some theoretical evaluations and in vitro testing. *J Control Release.* 1999;59:107-118.
28. Higuchi T. Physical chemical analysis of percutaneous absorption process from creams and ointments. *J Soc Cosmet Chem.* 1960;11:85-97.
29. Inatomi T, Spurr-Michaud S, Tisdale AS, Gipson IK. Human corneal and conjunctival epithelia express MUC1 mucin. *Invest Ophthalmol Vis Sci.* 1995;36:1818-1827.
30. Hidalgo IJ, Hillgren KM, Grass GM, Borchardt RT. Characterization of the unstirred water layer in Caco-2 cell monolayers using a novel diffusion apparatus. *Pharm Res.* 1991;8:222-227.
31. Wikman A, Karlsson J, Carlstedt I, Artursson P. A drug absorption model based on the mucus layer producing human intestinal goblet cell line HT29-H. *Pharm Res.* 1993;10:843-852.
32. Ohvo H, Slotte JP. Cyclodextrin-mediated removal of sterols from monolayers: effects of sterol structure and phospholipids on desorption rate. *Biochemistry.* 1996;35:8018-8024.