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In Vitro Assessment of Acyclovir Permeation Across Cell Monolayers in the Presence of Absorption Enhancers

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The aim of the investigation was to establish transepithelial permeation of acyclovir across Caco-2 and Madin-Darby canine kidney (MDCK) cell monolayers and attempt to improve its permeation by employing absorption enhancers (dimethyl β cyclodextrin, chitosan hydrochloride and sodium lauryl sulfate) and combinations thereof. Caco-2 and MDCK cell monolayers have been widely employed in studying drug transport, mechanisms of drug transport, and screening of absorption enhancers and excipients. Transepithelial electrical resistance and permeation of ^{99m}Tc-mannitol were employed as control parameters to assess the tight junction and paracellular integrity. Permeation of acyclovir in the presence of absorption enhancers was found to be significantly higher compared with drug permeation in their absence when assessed as apparent permeability coefficients (P_{app}). Synergistic improvements in P_{app} values of acyclovir were obtained in case-selected combinations of absorption enhancers; dimethyl β cyclodextrin–chitosan hydrochloride, chitosan hydrochloride–sodium lauryl sulfate, and dimethyl β cyclodextrin–sodium lauryl sulfate, were used. Recovery and viability assessment studies of both cell monolayers suggested reestablishment of paracellular integrity and no damage to cell membranes. Significantly improved permeation of acyclovir in the presence of selected combinations of absorption enhancers may be used as a viable approach in overcoming the problem of limited oral bioavailability of acyclovir.

Keywords acyclovir; absorption enhancers; permeation; Caco-2; MDCK; TEER; ^{99m}Tc-mannitol

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INTRODUCTION

Acyclovir (ACY; 9-[2-hydroxyethoxymethyl]-guanine) is a nucleoside analogue with potent antiviral activity for the herpes group of viruses (Elion et al., 1977; Schaeffer et al., 1978). For over two decades, ACY has been considered the first choice of treatment for herpes simplex virus (HSV) types 1 and 2 (HSV-1 and -2), but it has also been shown to be effective in the treatment of varicella-zoster, cytomegalovirus, and Epstein-Barr virus infections (De Clercq, 1993;

Jain et al., 2005). ACY is commercially available in intravenous, oral, and topical administrations (Stagni, Ali, & Weng, 2004). Although parenteral administration provides therapeutically effective blood levels, it is acceptable only in acute situations. In case of HSV infections, chronic therapy is needed, which leads to poor patient compliance and local toxicity at the injection site. Topical application of ACY is associated with very slow absorption (Jain et al., 2005). Oral administration of ACY is the most preferred route from the viewpoint of patient compliance and convenience. However, following oral administration, the absorption of the drug from the gastrointestinal tract is poor, slow, and variable. The oral bioavailability of ACY is low (10%–30%) and requires administration of 800 mg five times per day for the management of genital HSV disease (Tolle-Sander, Lentz, Maeda, Coop, & Polli, 2004; de Miranda & Blum, 1983). Immunocompetent patients who are already consuming a variety of other drugs fail to adhere to the dosing regimen of ACY. Also, low oral bioavailability is associated with greater intersubject variability of plasma concentrations and, hence, poorer control of the effects of ACY. In addition to this, there is drug wastage leading to economic disadvantage (Aungst, 2006). The reason for the low bioavailability of ACY following oral administration is its permeability limited absorption (BCS class III drug; Tolle-Sander et al., 2004). In addition to its low permeation, ACY is transported via passive diffusion through the paracellular route (de Vrueh, Smith, & Lee, 1998; Kristl & Tukker, 1998). As such the paracellular route occupies a very small surface area compared with the transcellular route and is sealed by tight junctions, which restrict the transport of hydrophilic molecules across the epithelium (Noach et al., 1994; Shah, Jogani, Bagchi, & Misra, 2006). One of the approaches to improve the permeability across the paracellular route is the coadministration of absorption enhancers like bile salts, surfactants, medium-chain fatty acids, cyclodextrins, and mucoadhesive polymers (Bernkop-Schnurch, 2000).

The aim of the investigation was to establish transepithelial permeation of ACY across Caco-2 and Madin-Darby canine kidney (MDCK) cell monolayers and attempt to improve its permeation by employing absorption enhancers (dimethyl β cyclodextrin [DM βCD], chitosan hydrochloride [CH], and sodium lauryl sulfate [SLS]) and combinations thereof to alleviate the problem of limited oral bioavailability of ACY. It was hypothesized that coadministration of ACY with absorption enhancers would lead to enhancement in permeation across the paracellular route. Increase in permeation of ACY across the intestinal epithelium may facilitate absorption of the drug across the intestinal epithelium, which in turn should lead to therapeutically effective systemic plasma levels and improvement in bioavailability.

Caco-2 (derived from human colorectal carcinoma) and MDCK (derived from the distal tubular part of the nephron of a dog kidney) cell monolayers have been widely employed in studying drug transport, mechanisms of drug transport, and screening of absorption enhancers and excipients (Shah et al., 2006; Irvine et al., 1999). These cells have been shown to possess morphological and functional similarity to intestinal enterocytes when cultured on semipermeable membranes. Two cell lines were used as they differ in their transepithelial electrical resistance (TEER) values, with MDCK cells having closer resemblance to the TEER values of the small intestine (30–100 $\Omega.cm^2$; Hidalgo, 2001).

MATERIALS AND METHODS

Materials

ACY was received as a gift from Alembic Limited (Vadodara, India). sodium pertechnetate was extracted from ^{99}Mo (supplied by the Regional Center for Radiopharmaceutical Division, Northern Region, Board of Radiation and Isotope Technology, Delhi, India) by using freshly distilled methyl ethyl ketone. Radionuclidic, radiochemical, and chemical purities were ensured before its use. CH (degree of deacteylation = 85%; molecular weight = 20 kDa) was purchased from S. A. Chemicals (Mumbai, India). Mannitol, stannous chloride dihydrate, acetic acid, sodium bicarbonate, DMβCD, SLS, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS; heat inactivated, U.S. origin), nonessential amino acids (NEAA), L-glutamine, benzyl penicillin, streptomycin, trypsin, ethylene diamine tetra acetic acid (EDTA), Hanks' Balanced Salt Solution (HBSS), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) and trypan blue were purchased from Sigma–Aldrich (Denmark). Culture plate inserts (0.4-μm pore size, 30-mm diameter, polycarbonate) were purchased from Millipore Corp. (Bedford, Massachusetts, USA).

Radiolabeling of Mannitol

Mannitol was labeled with ^{99m}Tc by a simple reduction method and optimized for maximum labeling efficiency (Eckelman, 1995; Mishra et al., 2004). Mannitol (10 mg) was dissolved in 1 ml of water for injection followed by the addition of 100 μg stannous chloride. The pH was adjusted to 6.0 ± 0.1 with 0.5 M sodium bicarbonate solution. The solutions were nitrogen-purged. The contents were filtered through a 0.22-μm membrane filter (Millipore Corporation, Bedford, Massachusetts, USA) into an evacuated sterile vial. One ml of sterile sodium pertechnetate (75–100 Mega Bequerel [MBq]) was added to the solution and mixed for 30 seconds. The labeling was carried out at room temperature (25 ± 2°C). The quality control of ^{99m}Tc-mannitol was performed using ascending instant thin layer chromatography (ITLC) using silica gel (SG) coated fiberglass sheets (Gelman Instrument Company, Ann Arbor, Michigan, USA) as per the method described by Mishra and colleagues (2004).

Maintenance of Cell Cultures

Caco-2 (passage 18) and MDCK cells (passage 31; subclone 2) were purchased from National Center for Cell Sciences (Pune, India). They were seeded at 1×10^4 cells/ml into 75 cm^2 tissue culture flasks (Costar Corporation, Cambridge, Massachusetts, USA). The Caco-2 cells were maintained in a culture medium consisting of DMEM containing 4.5 g/L glucose, supplemented with 10% v/v FBS, 1% v/v NEAA, 2 mM L-glutamine, 100 U/ml benzyl penicillin, and 100 µg/ml streptomycin, and the pH was adjusted to 7.40 using 0.5 M sodium bicarbonate solution. The MDCK cell culture medium additionally contained 1% v/v sodium pyruvate. Cells were maintained in a water-jacketed incubator (Forma Scientific, Ohio, USA) at 37°C and in an atmosphere of 5% CO_2. The culture media was changed three times per week. At 80% to 90% confluency, the cells were harvested by washing with Ca^{+2}/Mg^{+2}–free HBSS, followed by trypsinization (0.05% w/v trypsin and 0.02% w/v EDTA solutions for 5 minutes at 37°C), and were resuspended in their respective culture media. Cells were counted with a hemocytometer using a phase-contrast inverted microscope (Olympus Corporation, Tokyo, Japan). The cells were seeded onto polycarbonate inserts at a density of 2×10^5 cells/cm^2 and each insert was placed in an individual well of six well plates (Nunc, Roskilde, Denmark). Seeded cells were fed with fresh media three times per week. The growth of cells and degree of confluence was checked daily by microscopy and measurement of TEER. For permeation studies, Caco-2 (passages 31-42) and MDCK (passages 39–50) cells were used 18 to 24 days and 6 to 7 days postseeding, respectively.

Measurement of TEER

Resistance across cell monolayers was measured at 24-hour intervals until and after the cells attained confluence using a Millicell® ERS meter (Millipore, Bedford, Massachusetts, USA). TEER of cell monolayers was calculated by subtracting the background resistances obtained with blank wells. Increasing TEER readings indicate that cells were closely packed, forming tight junctions. Caco-2 cells were used at TEER values between 600–800 Ω.cm^2 and MDCK cells at 300 to 350 Ω.cm^2.

TEER values were measured during the permeation studies to ascertain the effect of absorption enhancer(s) at each sample point. The TEER values recorded at t = 0 hours were taken as initial values and expressed as 100%. The TEER values at each sampling point were expressed as percent of initial values.

Permeation Studies of ^{99m}Tc-mannitol

Permeation of ^{99m}Tc-mannitol, a paracellular marker (our unpublished data), across Caco-2 and MDCK cell monolayers was studied to assess paracellular integrity. Prior to the start of permeation studies, the culture media was removed from the apical (AP) and basolateral (BL) sides of the cell monolayers. Further, the cell monolayers were washed thrice with fresh prewarmed HBSS (37 ± 1°C), replaced with phosphate buffer saline, and equilibrated for 30 minutes.

Transepithelial permeation of ^{99m}Tc-mannitolwas carried out in AP to BL direction by placing 1 ml of ^{99m}Tc-mannitol (specific activity 7.5–10 MBq/mg, pH 6.3) on the AP side of the cell monolayers. The BL side contained 2.5 ml of phosphate buffer saline (pH 7.4). AP to BL permeation of ^{99m}Tc-mannitol in the absence (control) and presence of different concentrations of DMβCD (1%w/v, 3% w/v, and 5% w/v); CH (0.1% w/v, 0.3% w/v, and 0.5% w/v); SLS (0.009% w/v, 0.012% w/v, and 0.015% w/v); 5% w/v DMβCD–0.5% w/v CH, 0.5% w/v CH–0.015% w/v SLS; and 5% w/v DMβCD–0.015% w/v SLS combinations were studied. Samples (200 µL) were withdrawn from the receiver (BL) compartments at time intervals of 0, 0.5, 1.0, and 2.0 hours and replaced with an equal volume of fresh prewarmed (37 ± 1°C) phosphate buffer saline to maintain sink conditions. Samples withdrawn from BL compartments were analyzed immediately for the counts of radiomarker by a well-type gamma spectrometer (Capintec Inc., New Jersey, USA) consisting of a sodium iodide crystal doped with a very small amount of thallium (NaI[Tl]).

During the permeation of ^{99m}Tc-mannitol, the well plates were placed on a calibrated shaker at 50 rpm at 37°C so as to minimize the effects of the unstirred water layer.

Permeation Studies of ACY

Prior to the start of ACY permeation studies, Caco-2 and MDCK cell monolayers were equilibrated as mentioned in permeation studies of ^{99m}Tc-mannitol. Transepithelial permeation of ACY in AP to BL direction was carried out by placing 1 ml of 2.5 mM drug solution, prepared in HBSS + 10 mM HEPES (pH 6.3) on the AP side, and 2.5 ml of HBSS + 10 mM HEPES (pH 7.4) on the BL side. Transepithelial permeation of ACY in the presence of different concentrations of DMβCD (1% w/v, 3% w/v, and 5% w/v); CH (0.1% w/v, 0.3% w/v, and 0.5% w/v); and SLS (0.009% w/v, 0.012% w/v, and 0.015% w/v) were studied in AP to BL direction across both cell monolayers. In a similar manner, permeation of ACY was also studied in the presence of 5% w/v DMβCD–0.5% w/v CH, 0.5% w/v CH–0.015% w/v SLS, and 5% w/v DMβCD–0.015% w/v SLS combinations. Samples (200 µL) were withdrawn from BL compartments at different time intervals (0, 0.5, 1.0, and 2.0 hours) and replaced with an equal volume of BL medium. Withdrawn samples were stored at –20 °C prior to analysis by HPLC.

Permeation studies were carried out by placing the well plates on a calibrated shaker at 50 rpm at 37°C.

Drug Analysis

ACY was analyzed by an HPLC system (Dionex, GmbH, Germany) consisting of a Dionex P 680 LPG pump, a Dionex

UVD 170 UV/Visible multiple wavelength detector, and Chromeleon software (Version 6.50). The C-18 ODS Hypersil column (250 × 4.6 mm) with 10 µm particles of 120 Å pore size was employed. The mobile phase was water: acetic acid (99.9: 0.1) at a flow rate of 2.0 ml/minute and a detection wavelength of 254 nm.

Recovery Studies

After completion of the permeation studies of ^{99m}Tc-mannitol, the test solutions were removed carefully followed by slowly rinsing the cell monolayers thrice with fresh prewarmed HBSS (37 ± 1°C). Both AP and BL chambers of Caco-2 and MDCK cell monolayers were replenished with their appropriate culture media and replaced in an incubator at 37°C in an atmosphere of 5% CO_2. TEER values and transport of ^{99m}Tc-mannitol across both monolayers were determined at 24 hours posttreatment.

Assessment of Cell Viability

At the end of the permeation studies of ACY, the Caco-2 and MDCK cell monolayers were assessed for cell viability. Cells were removed from the inserts by trypsinization (0.05% w/v trypsin and 0.02%w/v EDTA). Trypsin activity was quenched by scrapping the cells into the respective culture medium. This was diluted with 1:1 trypan blue dye solution and cells were viewed under inverted microscope using a hemocytometer. Viable cells were detected by their ability to exclude trypan blue. The numbers of viable and nonviable cells were counted and percent cell viability was determined using the following Equation 1, and the cell viability of more than 90% was considered as nontoxic.

$$\%\ Cell\ Viability = \frac{Total\ Viable\ Cells\ (Unstained)}{Total\ Cells} \times 100 \quad (1)$$

Data Analysis

Apparent permeability coefficient (P_{app}) values of ^{99m}Tc-mannitol and ACY were calculated using Equation 2 and recorded in Tables 3, 4, and 5.

$$P_{app} = \frac{dQ}{dt} \times \frac{V_R}{A \times C_0} \quad (2)$$

Where,

P_{app} is the apparent permeability co-efficient (cm/s), dQ/dt is the cumulative flux in the AP to BL direction (µg/s), V_R is the volume of the receptor compartment (cm^3), A is the diffusion area of the monolayer (cm^2), and C_0 is the initial concentration applied on the AP side (µg).

All the values are expressed as mean ± standard deviation (*SD*) values. Statistical evaluation was carried out with one-way analysis of variance, and differences between groups at $p < .05$ were considered to be significant.

RESULTS

Effect of Absorption Enhancers on TEER of Caco-2 and MDCK Cell Monolayers

TEER values, obtained by treating Caco-2 and MDCK cell monolayers with different concentrations of DMβCD (1%–5% w/v), CH (0.1%–0.5% w/v), and SLS (0.009%–0.015% w/v), were recorded in Tables 1 and 2, respectively. In the case of control monolayers (i.e., without absorption enhancers), the TEER values at all time points did not change during the course of the experiments and were similar to the baseline (initial, t = 0 hours) values. Employment of absorption enhancers resulted in a significant ($p < .05$) decrease in TEER in a short time (t = 0.5 hours) in both monolayers. With time, further decrease in TEER was recorded, with the highest decrease at t = 2 hours. At all time points, the TEER values in both cell monolayers were significantly ($p < .05$) lower than their corresponding initial values. In the presence of 5% w/v DMβCD, 0.5% w/v CH, and 0.015% w/v SLS, TEER values reached 42%, 45%, and 63%, respectively, of initial values in Caco-2 cell monolayers at t = 2 hours (Table 1). In the case of MDCK cell monolayers, the presence of 5% w/v DMβCD, 0.5% w/v CH, and 0.015% w/v SLS resulted in TEER values reaching 39%, 41%, and 59% of initial values at t = 2 hours (Table 2).

Combinations of absorption enhancers also led to a significant ($p < .05$) reduction in TEER values in both cell monolayers. At all time points, the reduction caused due to the combination was higher than that caused by the single absorption enhancer. With 5% w/v DMβCD–0.5% w/v CH, 0.5% w/v CH–0.015% w/v SLS, and 5% w/v DMβCD–0.015% w/v SLS combinations, TEER values reached 27%, 30% and 31%, respectively, of the initial values in Caco-2 cell monolayers at t = 2 hours. In the case of MDCK cell monolayers, employment of these combinations resulted in TEER values reaching 26%, 28%, and 29% of initial values at t = 2 hours.

Permeation Studies of ^{99m}Tc-mannitol

^{99m}Tc-mannitol was employed for the assessment of paracellular integrity in Caco-2 and MDCK cell monolayers. Very low and negligible amounts of ^{99m}Tc-mannitol were transported across both monolayers in control groups (i.e., without absorption enhancers), as indicated by low P_{app} values. The P_{app} values of ^{99m}Tc-mannitol in Caco-2 and MDCK cell monolayers are recorded in Tables 3 and 4, respectively. P_{app} values of ^{99m}Tc-mannitol in control Caco-2 and MDCK cell monolayers were $0.286 \pm 0.056 \times 10^{-6}$ cm/seconds and $0.507 \pm 0.049 \times 10^{-6}$ cm/seconds, respectively.

TABLE 1
TEER Values in Caco-2 Cell Monolayers

Treatment	Time/TEER ($\Omega.cm^2$)*			
	0.5 hr	1 hr	2 hr	24 hr Posttreatment
Control	98.97 ± 2.11	98.21 ± 1.73	97.31 ± 1.86	98.59 ± 1.53
1% w/v DMβCD	62.51 ± 1.17*	58.32 ± 2.33#	54.45 ± 3.07#	97.74 ± 4.12
3% w/v DMβCD	58.93 ± 2.04#	55.36 ± 1.15#	46.74 ± 3.02#	96.85 ± 1.25
5% w/v DMβCD	59.07 ± 2.07#	47.3 ± 1.86#	42.33 ± 2.10#	95.78 ± 4.37
0.1% w/v CH	70.51 ± 4.12#	65.18 ± 2.22#	56.91 ± 1.97#	87.81 ± 1.38#
0.3% w/v CH	60.26 ± 2.04#	53.74 ± 3.55#	50.43 ± 1.77#	84.94 ± 2.29#
0.5% w/v CH	59.68 ± 2.45#	48.76 ± 1.94#	45.30 ± 3.01#	83.14 ± 1.75#
0.009% w/v SLS	83.40 ± 1.28#	74.10 ± 2.14#	71.35 ± 1.73#	97.36 ± 3.05
0.012% w/v SLS	78.65 ± 2.14#	70.35 ± 1.82#	66.56 ± 1.17#	97.27 ± 2.36
0.015% w/v SLS	78.69 ± 1.59#	70.34 ± 2.06#	63.38 ± 3.17#	96.57 ± 1.61
5% w/v DMβCD + 0.5% w/v CH	46.71 ± 1.40#	31.85 ± 1.72#	27.07 ± 1.65#	82.26 ± 2.48#
0.5% w/v CH + 0.015% w/v SLS	48.27 ± 3.01#	32.91 ± 2.18#	29.84 ± 1.33#	83.08 ± 1.54#
5% w/v DMβCD + 0.015% w/v SLS	49.31 ± 1.35#	35.59 ± 1.92#	31.19 ± 1.37#	97.66 ± 2.05

*TEER values are expressed as percent of initial values.
#$p < .05$; significant difference from control.
All values expressed as $M \pm SD$ ($n = 6$).

TABLE 2
TEER Values in MDCK Cell Monolayers

Treatment	Time/TEER ($\Omega.cm^2$)*			
	0.5 hr	1 hr	2 hr	24 hr Posttreatment
Control	98.72 ± 1.96	97.91 ± 2.17	96.83 ± 1.42	98.79 ± 3.08
1% w/v DMβCD	61.63 ± 1.51#	51.66 ± 1.39#	45.92 ± 1.64#	96.68 ± 2.15
3% w/v DMβCD	60.12 ± 3.11#	48.16 ± 2.43#	42.33 ± 3.02#	94.79 ± 1.79
5% w/v DMβCD	56.68 ± 1.59#	43.92 ± 2.75#	38.58 ± 1.36#	93.75 ± 2.65
0.1% w/v CH	68.21 ± 2.05#	64.20 ± 1.73#	53.40 ± 1.82#	82.72 ± 1.36#
0.3% w/v CH	67.29 ± 1.72#	56.07 ± 2.02#	43.93 ± 1.57#	81.31 ± 1.33#
0.5% w/v CH	64.08 ± 4.02#	53.40 ± 2.35#	41.42 ± 2.46#	80.26 ± 1.38#
0.009% w/v SLS	88.87 ± 3.06#	76.83 ± 2.24#	68.57 ± 1.28#	96.19 ± 1.47
0.012% w/v SLS	80.12 ± 1.93#	72.78 ± 1.26#	62.69 ± 2.08#	95.80 ± 2.57
0.015% w/v SLS	79.26 ± 2.23#	70.90 ± 1.45#	59.13 ± 2.14#	94.91 ± 3.06
5% w/v DMβCD+0.5% w/v CH	45.47 ± 2.29#	30.00 ± 1.87#	26.18 ± 1.57#	79.89 ± 2.6#
0.5% w/v CH+0.015% w/v SLS	47.35 ± 1.76#	32.11 ± 1.80#	27.80 ± 1.95#	79.57 ± 2.24#
5% w/v DMβCD+0.015% w/v SLS	49.05 ± 1.71#	33.69 ± 1.34#	29.21 ± 2.46#	93.44 ± 3.13

*TEER values expressed as percent of initial values.
#$p < .05$; significant difference from control.
All values expressed as $M \pm SD$ ($n = 6$).

In the presence of absorption enhancers DMβCD, CH, and SLS, significantly ($p < .05$) higher P_{app} values of ^{99m}Tc-mannitol were recorded in both cell monolayers as compared with control groups. The increments in the P_{app} values of ^{99m}Tc-mannitol in the presence of absorption enhancers were concentration dependent. 5% w/v DMβCD, 0.5% w/v CH, and 0.015% w/v SLS led to 11-, 9-, and 6-fold increases, respectively, in P_{app} values of ^{99m}Tc-mannitol as compared

with the control in Caco-2 cell monolayers. When 5% w/v DMβCD–0.5% w/v CH, 0.5% w/v CH–0.015% w/v SLS, and 5% w/v DMβCD–0.015% w/v SLS combinations were employed, the increments in P_{app} values of ^{99m}Tc-mannitol were 27-, 20-, and 21-fold increase, respectively, as compared with the control. In MDCK cell monolayers, 5% w/v DMβCD, 0.5% w/v CH, and 0.015% w/v SLS led to 14-, 13-, and 8-fold increases, respectively, in P_{app} values of ^{99m}Tc-mannitol as compared with the control group, whereas 5% w/v DMβCD–0.5% w/v CH, 0.5% w/v CH–0.015% w/v SLS, and 5% w/v DMβCD–0.015% w/v SLS combinations led to 34-, 26-, and 28-fold increases, respectively, P_{app} values of marker molecule.

Permeation Studies of ACY

Transepithelial permeation of ACY in AP to BL direction was studied. The P_{app} values of ACY are recorded in Table 5. In the control (i.e., without absorption enhancers) Caco-2 and MDCK cell monolayers, P_{app} values of ACY were $0.352 \pm 0.07\times10^{-6}$ cm/seconds and $0.523 \pm 0.011\times10^{-6}$ cm/seconds, respectively. The influence of individual absorption enhancers DMβCD (1%–5% w/v), CH (0.1%–0.5% w/v), and SLS (0.009%–0.015% w/v) on ACY permeation across Caco-2 and MDCK cell monolayers was studied. Significantly ($p < .05$) higher P_{app} values of ACY (Table 5) as compared with control groups were recorded in Caco-2 and MDCK cell monolayers. A concentration-dependent effect of absorption enhancers on permeability of ACY was recorded across both cell monolayers (Table 5). In the presence of 5% w/v DMβCD, 0.5% w/v CH, and 0.015% w/v SLS, P_{app} values of ACY were increased 11-, 10-, and 6-fold, respectively as compared with the control in Caco-2 cell monolayers. The corresponding increment values in MDCK cell monolayers were 15-, 14-, and 8-fold, increase, respectively.

The P_{app} values of ACY were also studied in the presence of combinations of absorption enhancers across both cell monolayers. 5% w/v DMβCD–0.5% w/v CH, 0.5% w/v CH–0.015% w/v SLS, and 5% w/v DMβCD–0.015% w/v SLS increased the P_{app} values of ACY 27-, 20-, and 22-fold, respectively, as compared with the control in the Caco-2 cell monolayers. These combinations were responsible for 37-, 26-, and 27-fold increments increase, respectively, across MDCK cell monolayers.

Recovery Studies

TEER and permeation of ^{99m}Tc-mannitol across Caco-2 and MDCK cell monolayers 24 hours posttreatment were determined to check the reversibility of the effect of the absorption enhancers. In cell monolayers treated with all concentrations of DMβCD and SLS as well as the DMβCD–SLS combination, TEER values recovered and reached to > 90% of initial values within 24 hours posttreatment (Tables 1 and 2). Similarly, the P_{app} values of ^{99m}Tc-mannitol had recovered to almost control values (Tables 3 and 4). However, in the case of CH, DMβCD–CH and CH–SLS combination treated monolayers, TEER (Tables 1 and 2) and P_{app} values of ^{99m}Tc-mannitol (Tables 3 and 4) exhibited ≈ 80% recovery compared with control values across both cell monolayers.

Assessment of Cell Viability

After the permeation studies, the viability of Caco-2 and MDCK cells was determined using the trypan blue staining. The viability of Caco-2 and MDCK cells treated with absorption enhancers and combinations was > 90% and not significantly ($p > .05$) different from the control (i.e., without absorption enhancer) cells.

DISCUSSION

ACY is known to possess permeability-limited absorption (Tolle-Sander et al., 2004). For improving, paracellular permeation of ACY, we employed DMβCD (1%–5% w/v), CH (0.5%–1.5% w/v), and SLS (0.009%–0.015% w/v) as absorption enhancers. These agents are known to act by the following mechanisms. Cyclodextrins lead to absorption enhancement probably by the opening of the tight junctions, extracting the phospholipids and proteins from the membrane, thereby transiently altering membrane permeability and by overcoming the aqueous diffusion barrier (Masson, Loftsson, Masson, & Stefansson, 1999; Hovgaard & Brondsted, 1995). Chitosan, due to its bioadhesive nature, provides greater time for contact with the mucosa. The positive charge of the polymer (due to an amino group at the C-2 position) interacts with the negatively charged sialic groups sites of the glycoproteins on the surface of epithelial cells and tight junctions via electrostatic interactions (Artursson, Lindmark, Davis, & Ilium, 1994; Kerec, Bogataj, Veranic, & Mrhar, 2005). Once this polymer binds to the epithelium, it induces changes in distribution of cytoskeletal F-actin, occludin, and zonula occludens-1 (ZO- 1). This follows the opening of the tight junctions, leading to enhanced transport via the paracellular pathway (Artursson et al., 1994; Ranaldi, Marigliano, Vespignani, Perozzi, & Sambuy, 2002; Smith, Wood, & Dornish, 2004; Dodane, Khan, & Merwin, 1999; Schipper et al., 1997; Thanou, Verhoef, & Junginger, 2001; Junginger & Verhoef, 1998). SLS has been reported to improve paracellular transport by causing changes in the tight junctional structure and altering membrane permeability (Canfield, Geerdes, & Molitoris, 1991).

The concentrations of absorption enhancers employed in this study were based on the previous work carried out in our laboratory and reported work using these absorption enhancers (Udata et al., 2003; Kotze et al., 1999; Anderberg & Artursson, 1993).

Effect of Absorption Enhancers on TEER of Caco-2 and MDCK Cell Monolayers

TEER measurement is a simple, quick and easy tool for measurement of tight junction integrity (Kotze et al., 1998; Noach et al., 1994). TEER measurements of Caco-2 cell monolayers were higher than the values for MDCK cell monolayers, suggesting greater tightness of Caco-2 cell monolayers.

TEER measurements in control Caco-2 and MDCK cell monolayers, at all time points, exhibited values similar to the initial values (Tables 1 and 2) suggesting integrity of tight junctions. In the presence of absorption enhancers, the TEER values were found to be significantly lower ($p < .05$) than the initial baseline values in both cell monolayers. This may be explained by the modulation of tight junctions caused by these enhancers. The reductions in TEER values in both cell monolayers were more pronounced in DMβCD and CH treated monolayers compared with the values obtained using SLS as an absorption enhancer.

As shown in Tables 1 and 2, a significant reduction ($p < .05$) in TEER values was observed for both cell lines comparing the lowest concentration of each enhancer (1% w/v DMβCD, 0.1% w/v CH, and 0.009% w/v SLS) with the corresponding intermediate concentration (3% w/v DMβCD, 0.3% w/v CH, and 0.012% w/v SLS). Regarding the highest concentrations of the enhancers tested (5% w/v DMβCD, 0.5% w/v CH, and 0.015% w/v SLS), a different trend was observed for the two cell lines. A concentration-depending reduction of TEER values across both cell monolayers was observed only using DMβCD as an enhancer. With regards to CH, there was no statistical difference between 0.3% CH and 0.5% CH, both at t = 1 hour and at t = 2 hours measuring TEER values in MDCK cells. The use of 0.015% w/v of SLS did not provide any further reduction in TEER values compared with the lower concentrations of SLS for both cell lines. The different behavior of the enhancers tested could be attributed to their different water solubility and/or to a different tight junction modulation depending on the cell line involved. In the presence of a combination of enhancers, the TEER values were also significantly lower ($p < .05$) than the initial baseline values.

Permeation Studies of ^{99m}Tc-mannitol

Assessment of paracellular integrity is an important aspect while studying the paracellular transport. TEER values do not provide any information regarding the paracellular integrity (Mukherjee, Squillantea, Gillespieb, & Shao, 2004). Negligible amounts of ^{99m}Tc-mannitol transport in control groups of Caco-2 and MDCK cell monolayers suggested that the paracellular integrity was not compromised. The P_{app} values of ^{99m}Tc-mannitol were lower in Caco-2 cell monolayers compared with MDCK cell monolayers (Tables 3 and 4). This is due to the greater resistance offered by Caco-2 cell

TABLE 3
Apparent Permeability Coefficients (P_{app}) of ^{99m}Tc-Mannitol Across Caco-2 Cell Monolayers

	P_{app} in Caco-2 ($\times 10^{-6}$ cm/sec)	
Treatment	2 hr	24 hr Posttreatment
Control	0.286 ± 0.056	0.289 ± 0.037
1% w/v DMβCD	2.057 ± 0.066*	0.304 ± 0.032
3% w/v DMβCD	2.399 ± 0.038*	0.305 ± 0.081
5% w/v DMβCD	3.103 ± 0.126*	0.309 ± 0.064
0.1% w/v CH	1.708 ± 0.097*	0.333 ± 0.030*
0.3% w/v CH	2.42 ± 0.124*	0.348 ± 0.055*
0.5% w/v CH	2.655 ± 0.045*	0.351 ± 0.070*
0.009% w/v SLS	0.718 ± 0.092*	0.291 ± 0.022
0.012% w/v SLS	1.187 ± 0.106*	0.299 ± 0.031
0.015% w/v SLS	1.605 ± 0.110*	0.297 ± 0.125
5% w/v DMβCD+ 0.5% w/v CH	7.594 ± 0.388*	0.356 ± 0.065 *
0.5% w/v CH + 0.015% w/v SLS	5.700 ± 0.332*	0.347 ± 0.050*
5% w/v DMβCD+ 0.015% w/v SLS	6.115 ± 0.281*	0.301 ± 0.048

*$p < .05$; significant difference from control.
All values expressed as $M \pm SD$ ($n = 3$).

TABLE 4
Apparent Permeability Coefficients (P_{app}) of ^{99m}Tc-mannitol Across MDCK Cell Monolayers

	P_{app} in MDCK ($\times 10^{-6}$ cm/sec)	
Treatment	2 hr	24 hr Posttreatment
Control	0.507 ± 0.049	0.505 ± 0.102
1% w/v DMβCD	5.669 ± 0.229*	0.520 ± 0.061
3% w/v DMβCD	6.891 ± 0.155*	0.527 ± 0.072
5% w/v DMβCD	7.317 ± 1.089*	0.526 ± 0.046
0.1% w/v CH	3.542 ± 0.294*	0.570 ± 0.066*
0.3% w/v CH	5.335 ± 0.333*	0.585 ± 0.048*
0.5% w/v CH	6.88 ± 0.089*	0.591 ± 0.079*
0.009% w/v SLS	2.059 ± 0.056*	0.511 ± 0.038
0.012% w/v SLS	3.226 ± 0.128*	0.518 ± 0.029
0.015% w/v SLS	4.014 ± 0.176*	0.522 ± 0.086
5% w/v DMβCD+ 0.5% w/v CH	17.237 ± 1.125*	0.600 ± 0.058*
0.5% w/v CH+ 0.015% w/v SLS	13.211 ± 0.906*	0.594 ± 0.092*
5% w/v DMβCD+ 0.015% w/v SLS	14.20 ± 0.884*	0.532 ± 0.77

*$p < .05$; significant difference from control.
All values expressed as $M \pm SD$ ($n = 3$).

monolayers in comparison with MDCK cell monolayers. This is in agreement with TEER values across the two cell monolayers.

Significantly, higher P_{app} values for ^{99m}Tc-mannitol in the presence of DMβCD, CH, and SLS compared with control groups may be attributed to the modulation of the paracellular pathway caused by these absorption enhancers. This compromised paracellular integrity led to greater permeation of ^{99m}Tc-mannitol across the cell monolayers.

When combinations of two absorption enhancers were employed, synergistic rather than additive increase in the permeation of ^{99m}Tc-mannitol was observed (Tables 3 and 4). The synergistic improvement may be explained on the basis of different mechanisms of modulation of the paracellular pathway when these absorption enhancers were employed simultaneously. In the case of TEER values, no synergistic or additive reduction was observed (Tables 1 and 2). This may be because TEER values could only account for tight junction modulation and not paracellular modulation (Mukherjee et al., 2004). These enhancers also act by mechanisms other than tight junction modulation (Masson et al., 1999; Hovgaard & Brondsted, 1995; Thanou et al., 2001; Junginger & Verhoef, 1998). It has also been shown by Balda and colleagues (1996) that TEER and mannitol permeation are not always correlated. The reduction in TEER values is not the only factor responsible for the improvement of drug permeability (Mukherjee et al., 2004). Drug permeation may also occur by intercellular pathway. Hence, although the effects of combinations of two enhancers on TEER values were neither synergistic nor additive, each combination of enhancers tested worked better than a single enhancer.

Permeation Studies of ACY

In vitro drug permeation studies of ACY were carried out from the AP to BL side in order to predict the absorption of compounds from the lumen of the gut (i.e., mucosal to serosal side, absorptive transport). Very low and negligible P_{app} values of ACY observed in control groups (i.e., without absorption enhancer; Table 5) of both cell monolayers is due to the fact that ACY exhibits poor permeation characteristics (BCS Class III drug). It is transported across the epithelium via the paracellular route (de Vrueh et al., 1998; Kristl & Tukker, 1998). In the case of control Caco-2 and MDCK cell monolayers where the paracellular integrity is intact, the drug is transported only in small amounts across the epithelium. Low P_{app} values of ACY have also been reported by Irvine and colleagues (1999) and Taub and colleagues (2002). As expected, the P_{app} values of ACY were lower in Caco-2 cell monolayers as compared with MDCK cell monolayers due to the greater resistance offered by Caco-2 cell monolayers in comparison with MDCK cell monolayers.

Significant ($p < .05$) enhancements in P_{app} values of ACY in the presence of different absorption enhancers (Table 5) compared with control groups may be explained as follows: DMβCD, CH, and SLS acting through different mechanisms cause modulation of the paracellular pathway, which is responsible for greater amounts of drug permeating the epithelium and reaching the BL compartment. The enhancements obtained in P_{app} values were higher in MDCK cell monolayers compared with Caco-2 cell monolayers due to the greater tightness of Caco-2 cell monolayers. A similar trend was also observed during permeation studies of ^{99m}Tc-mannitol.

The P_{app} values of ACY in the presence of combinations of absorption enhancers (Table 5) in both cell monolayers were higher than the P_{app} values of individual absorption enhancers. Synergistic improvements in P_{app} values were observed in both cell monolayers when combinations of absorption enhancers were employed. The synergistic effect in drug permeation may be attributed to more than one mechanism involved, leading to greater than additive effect of the two individual absorption enhancers. The highest increments in P_{app} values of ACY were recorded with the 5% w/v DMβCD–0.5% w/v CH combination. The synergism between chitosan and cyclodextrin may be explained as follows. When chitosan interacts with the epithelial membrane, the tight junctions are opened, then cyclodextrins are able penetrate into the opened gaps between cells and extract the

TABLE 5
Apparent Permeability Coefficients (P_{app}) of ACY Across Caco-2 and MDCK Cell Monolayers

Treatment	P_{app} in Caco-2 ($\times 10^{-6}$ cm/sec)	P_{app} in MDCK ($\times 10^{-6}$ cm/sec)
Control	0.352 ± 0.07	0.523 ± 0.011
1% w/v DMβCD	2.396 ± 0.261*	5.822 ± 0.169*
3% w/v DMβCD	3.093 ± 0.142*	6.953 ± 0.217*
5% w/v DMβCD	3.816 ± 0.34*	7.896 ± 0.412*
0.1% w/v CH	2.04 ± 0.149*	3.75 ± 0.677*
0.3% w/v CH	3.169 ± 0.101*	5.718 ± 0.851*
0.5% w/v CH	3.591 ± 0.096*	7.239 ± 0.519*
0.009% w/v SLS	0.771 ± 0.114*	1.744 ± 0.159*
0.012% w/v SLS	1.47 ± 0.213*	2.96 ± 0.156*
0.015% w/v SLS	1.986 ± 0.083*	3.957 ± 0.206*
5% w/v DMβCD+ 0.5% w/v CH	9.513 ± 0.389*	19.147 ± 0.614*
0.5% w/v CH+ 0.015% w/v SLS	7.181 ± 0.214*	13.531 ± 0.497*
5% w/v DMβCD+ 0.015% w/v SLS	7.789 ± 0.282*	13.877 ± 0.356*

*$p < .05$; significant difference from control.
All values expressed as $M \pm SD$ ($n = 3$).

phospholipids in the biomembrane. Thus, the tight junction proteins such as occludin (Furuse et al., 1993), claudin-1 and -2 (Furuse, Fujita, Hiiragi, Fujimoto, & Tsukita, 1998) are naked and may collapse after the removal of surrounding phospholipids, resulting in these fusion points coming untied. Therefore, the opening of the tight junctions may be strengthened by coadministration of chitosan and cyclodextrins.

Recovery Studies

Absorption enhancers are expected to modulate the paracellular pathways in a reversible and safe manner, which is an important issue to be studied during screening of such agents. Complete recovery of cells is highly desirable before they are exposed to the next dose of absorption enhancers to avoid cell damage due to repeated administration (Tirumalasetty & Eley, 2005, 2006). Lack of recovery of barrier properties after treatment of an in vivo epithelium could lead to problems such as increased risk of infection of the area. In cell monolayers treated with DMβCD and SLS, as well as the DMβCD–SLS combination, reformation of tight junctions upon removal of these agents from contact with the monolayers was observed as indicated by >90% recovery of TEER values within 24 hours. Similarly, regaining paracellular integrity was achieved as indicated by almost similar P_{app} values of the marker molecule 24 hours posttreatment as those of to control values. However, in the case of CH-treated cell monolayers and combinations containing CH, the recovery of TEER and P_{app} values of ^{99m}Tc-mannitol was ≅ 80%. The slightly lower recovery in the case of CH-treated cell monolayers may be explained by the mucoadhesive nature and viscosity of CH (Lehr, Bouwstra, Schacht, & Junginger, 1992), due to which complete removal of CH from the cell surface is not possible.

Assessment of Cell Viability

After treating the cell monolayers with DMβCD, CH, and SLS, viability of the cells was assessed to confirm whether or not the permeation enhancement caused by these agents was due to loss of cell viability. The monolayer that had no enhancer employed was assigned as control. For the cell viability assessment, a diazo dye, trypan blue, was employed. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable (Freshney, 1987). Caco-2 and MDCK cell monolayers treated with absorption enhancers and combinations had viability similar to the controls, suggesting that the permeation enhancement caused by them was not due to any loss of cell viability.

CONCLUSION

The transepithelial permeation of ACY across Caco-2 and MDCK cell monolayers in the presence of absorption enhancers was found to be significantly higher compared with drugs without absorption enhancers. Synergistic improvements in the permeation of ACY were observed when selective combinations of DMβCD–CH, CH–SLS, and DMβCD–SLS were used. Hence, to achieve the similar enhancement in the permeation of ACY, smaller concentrations of individual absorption enhancers will be required, and it will minimize toxicities of individual absorption enhancers. To conclude, coadministration of these absorption enhancers in ACY oral formulations may lead to an improvement in absorption and bioavailability. To use the concept reported in this paper inclinical practice, extensive pharmacokinetic evaluation in animals followed by clinical investigations is required.

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