Effects of Inclusion Complexation on the Transepithelial Transport of a Lipophilic Substance *in Vitro*

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Poor oral bioavailability of three experimental compounds, I ~ III, observed in animals has been attributed to the low intrinsic solubility. To enhance their GI absorption, we attempted to increase the solubility of these compounds with hydroxypropyl β-cyclodextrin (HPB)[4] and γ-cyclodextrin (HPG). Compound I showed an increase in solubility over 1,000-fold with 25% HPB at 25°C. The association constant of the 1:1 complex between I and HPB was determined by phase-solubility analysis. Thermodynamic parameters involved were all favorable for the complexation. The large positive $\Delta S°$ observed suggests that the complex formation is driven by a hydrophobic interaction. Apical-to-basal transport of I across the Madin Darby canine kidney (MDCK) cell monolayer was studied at 37°C in the presence of HPB with or without agitation. The complex itself did not pass through the cell layer. Diffusion of the unbound I as well as the complex through the aqueous boundary layer in the apical side is rate-limiting. Regardless of hydrodynamics, decreasing HPB concentration at a given drug concentration increased the transport rate. The findings indicate that the transepithelial transport is attributed to the passive diffusion of available free drug molecules rather than the collision complex transfer at the cell surface.

KEY WORDS: complexation; membrane transport; free drug concentration; hydroxypropyl cyclodextrins; chromone derivatives.

INTRODUCTION

Water-insoluble lipophilic substances frequently show limited oral bioavailability as a consequence of absorption-limiting slow dissolution in the GI lumen, high first-pass hepatic extraction following the absorption, or the combination of both (1). Experimental chromone derivatives, I–III, are potent inhibitors of smooth muscle cell proliferation *in vitro*. However, they show poor bioavailability in rats and rabbits when orally administered in suspensions (2). Their intrinsic solubilities range from 2.4 μg/ml for I to 12.5 μg/ml for II. Although a significant first-pass effect cannot be ruled out, presence of undissolved solids in the gut lumen one day after dosing supported that the poor oral bioavailability observed could be due to low solubility and slow dissolution of the compounds. Given these observations, soluble complexes of these compounds were believed to enhance the dissolution rate and possibly the GI absorption.

Effects of complexation on membrane transport have been a subject of several excellent investigations, especially when the complexing ligand involved is a macromolecule (e.g., 3,4). In contrast, it is not clear how a molecular interaction with a small molecular ligand affects the transepithelial transport of a lipophilic solute. Plasma phenytoin level in dogs subsequent to oral administration of the drug as a complex with a β-cyclodextrin derivative was found to be approximately twice that observed without the complexing ligand (5). Absorption of water-insoluble steroids through sublingual/buccal (6) and nasal (7) epithelium was much greater when given as inclusion complexes. In these studies, for example, it was implied but not clearly stated that the complexes themselves are not absorbed and that only free drug molecules dissociated from the complexes are the species subject to transport.

In this report, the molecular interaction between I–III and hydroxypropyl cyclodextrins is characterized by means of phase-solubility analysis (8). Various water-soluble cyclodextrin derivatives (9–12) form an inclusion complex with a solute of appropriate size and hydrophobicity, frequently resulting in a profound change in physical and chemical properties of the guest molecule (13,14). We then report the effect of hydroxypropyl β-cyclodextrin (HPB) on the transepithelial transport of I across a Madin Darby canine kidney (MDCK) cell monolayer grown on a porous membrane. The latter consists of two chambers separated by the cell monolayer grown on a polycarbonate membrane, 2.45 cm in diameter, with 3- or 0.4-μ pores. The system was characterized with polar permeants such as sucrose (15) and a series of aliphatic carboxylic acids (16), used in testing a hypothesis on the role of citric acid in GI absorption of peptides (17), and in exploring a possibility of using leukocytes in targeted drug delivery (18). Unlike the commonly used Caco-2 cell monolayer (19), the MDCK cells do not require a long incubation period for growth to confluence. So long as a transport phenomenon of research interest does not involve an active process, as in the present study, the system serves as an excellent model alternative to the Caco-2 cell monolayer and whole animal experiments.

MATERIALS AND METHODS

Materials

8-Methyl-2-(4-morpholinyl)-7-(phenylmethoxy)-4H-1-benzopyran-4-one (I), 8-methyl-2-(4-morpholinyl)-7-(2-pyridinylmethoxy)-4H-1-benzopyran-4-one (II), and 8-[(4-ethoxyphenyl) methoxy]-2-(4-morpholinyl)-4H-1-benzopyran-4-one (III), were obtained from the Upjohn Laboratories and used as received. HPB, HPG, and [^{14}C]HPB were purchased from Cyclodextrin Technol. Dev. Inc. (Gainesville, FL; formerly Pharmatec, Inc.). [^{14}C]Sucrose and [^{125}I]-bovine serum albumin (BSA) were obtained from New England Nuclear (Boston, MA). The latter was subject to Centricon® 30(Amicon/Danver, MA) centrifugation to remove small MW impurities (16). Tissue culture media and

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[4] Abbreviations used: A, apical; B, basal or basolateral; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; HPB, hydroxypropyl β-cyclodextrin; HPG, hydroxypropyl γ-cyclodextrin; MDCK, Madin Darby canine kidney.

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culture plates used for routine maintenance of cell monolayers were described elsewhere (15,16).

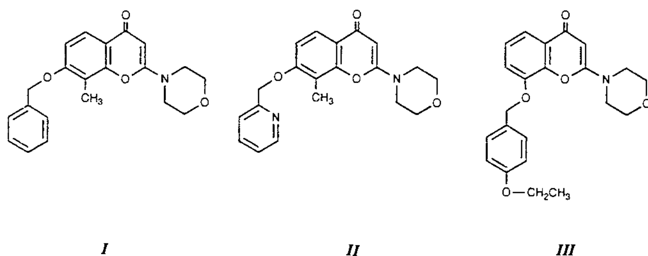
Solubility Determinations

Intrinsic solubilities of I–III were determined in distilled water. Solubility equilibrium was attained by continuously shaking the samples for at least one week. Apparent solubility in the presence of HPB or HPG was measured in 2.0 ml using a 3.5 ml vial (Pierce; Rockford, IL). For convenience, a 25% stock solution was prepared and properly diluted prior to experiments, all in distilled water. At equilibrium samples were filtered by either medium-porosity sintered glass filters or 0.45 μ Millex-HV filters (Millipore; Bedford, MA). In either case, about 0.5 ml of the initial filtrate was discarded. Phase solubility analysis of I was performed also at 4, 25, 37, and 47°C at HPB concentrations of 2, 4, 6, 8, and 10%. In these experiments, care was taken to pre-equilibrate all filters and their accessories at the appropriate temperature. To facilitate dissolution, samples at 4 and 25°C were first shaken at 47°C for one day and further shaken at the final temperatures for another two days. During the attainment of solubility equilibrium, the compounds were stable, as evidenced by single spot on TLC of ethyl acetate extract of the final filtrates, or by single peak in reversed-phase HPLC elution profiles.

In all cases, the drug concentration in the final filtrate, after dilution in distilled water if necessary, was determined from UV absorption. Absorption maximum occurs in the vicinity of 320 nm for all compounds. Their UV spectra were not altered by the presence of HPB or HPG after dilution. UV absorption from HPB or HPG in the diluted samples were negligible and therefore ignored. Extinction coefficient of I–III was determined in 50% acetonitrile and assumed to be the same in the water. UV absorption was measured using a Perkin Elmer model Lambda 6 spectrophotometer (Norwalk, CT).

Transport Experiments

Transport of I across the cell monolayer in the presence of HPB was studied with or without rocking the system. Preparation of MDCK cell monolayer and basic procedure of transport experiment were previously described in detail (15,16). Briefly, the cells were seeded at 7.5×10^4 cells/cm^2 on a 0.4 μ Transwell® (Costar, Cambridge, MA) in a culture medium containing 10% fetal bovine serum and grown to confluence under 5% CO_2 atmosphere at 37°C. On day 5, the medium was replaced with 37°C Hank's balanced salt solution (HBSS). After 60 min, a transport experiment was initiated by completely replacing 1.5 ml apical HBSS with a drug-containing test solution in HBSS with varying amount of HPB that had been pre-equilibrated at 37°C. The apical to basolateral (A to B) transport was continuously followed by monitoring the appearance of I in 2.5 ml HBSS in the basal receiving side. Throughout the study, the latter always contained 0.25% BSA to accommodate I transported. To maintain a sink condition, the Transwell® with the cell monolayer containing a test solution was moved to a fresh HBSS in the next well of a 6-well plate every hour. When agitation of the system was desired, the 6-well plate was continuously swirled on a cell rocker (Clay Adams, Parsippany, PA) inside a cell incubator.



Concentration of I was determined from UV absorption, using control HBSS containing 0.25% BSA as a reference. For convenience, the absorbance at 318 nm of all accumulated samples was read overnight by means of an autosampler/sipper system (Perkin Elmer). At the end of a given experiment, mass balance was determined by summing up all mass transported and that remained in the donor side. In most cases, the total recovery was in the range of 95 to 105%. In some experiments, the A chamber was spiked with [^{14}C]-sucrose or [^{14}C]-HPB. In other experiments, the B side contained [^{125}I]-BSA. In the former case, the appearance of radioactivity in the B medium was determined. In the latter case, total radioactivity from [^{125}I]-BSA accumulated in the A side was determined at the end of a given transport experiment.

Viscosity Adjustment

In some transport experiments, a HPB concentration as high as 10% was employed. The viscosity of other test solutions where total HPB concentration was less than 10% was adjusted to that of 10% HPB using 25% Ficoll® 400 (Pharmacia; Piscataway, NJ). A Cannon-Fenske viscometer (Scientific Products; McGaw Park, IL) was calibrated with distilled water in a water bath at 37 ± 0.2°C. Density of HPB or Ficoll® 400 solutions in HBSS at 37°C was determined using 10-ml volumetric flasks of known tare in a 37°C water bath. Experimental details are described elsewhere (20). It was assumed throughout the study that Ficoll® 400 does not undergo any molecular interaction with I.

RESULTS AND DISCUSSIONS

Molecular Interaction Between I–III and Hydroxypropyl Cyclodextrins

Increase in apparent solubility of I ~ III in water at 25°C is shown in Figure 1A as a function of HPB concentration and in Figure 1B as a function of HPG concentration. For all compounds studied, HPB increased the solubility more than HPG. As shown in Table I, at 25%, HPB was about 10-, 5-, and 6-fold better than HPG for I, II, and III, respectively. This difference in solubilizing capacity could well be due to the difference in the cavity size of the host molecules. The number of D-glycosyl repeating units is 7 and 8 for naturally occurring β- and γ-cyclodextrin, providing cavity widths of 6.0–6.4 and 7.5–8.3 Å, respectively (14). With HPB, the ranking of solubility enhancement was I > III > II. For example in a 25% HPB solution, the increase in solubility was approximately 1,200-, 130-, and 100-fold, respectively.

The three chromone compounds we studied structurally

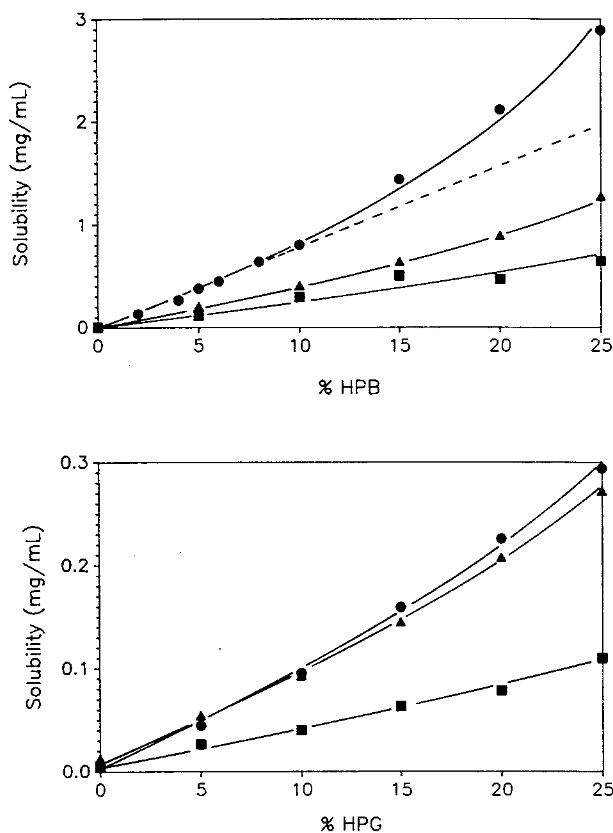


Fig. 1. Apparent aqueous solubility at 25°C of I (circles), II (triangles), and III (squares) in HPB (Fig. 1A; upper panel) and HPG (Fig. 1B; lower panel) solutions.

differ from one another only in the substitution at C7 or C8 of the common skeleton. However, they showed significant differences in affinity towards HPB or HPG resulting in different apparent aqueous solubility. These observations led us to speculate that the benzyl, pyridylmethyl, and *p*-ethoxybenzyl moieties in I - III are likely inserted in the cavity of the host molecule. Substitution of the phenyl group in I with a pyridyl moiety in II is expected to decrease the lipophilicity, which could result in a significant decrease in hydrophobic interaction. The *para*-ethoxy substitution in III may interfere with the maximal inclusion of the benzyl moiety resulting in a reduced interaction with HPB or HPG.

The 1:1 interaction implied above between a guest and a host molecule is supported by the linear increase in solubility observed with the cyclodextrin derivatives for their concentration up to approximately 10% (Fig. 1). Such a phase diagram, commonly referred to as A_L-type, is indicative of complexes of the first order in the ligand, HPB or HPG in the present case. With the possible exception of III, in all cases the solubility increase was non-linear especially when HPB or HPG concentration was greater than 10%. The positive deviation, A_p-type, is generally attributed to step associations involving various complexes in which more than one host molecule interact with one guest host molecule (8). In the present study, no attempt was made to further characterize the system with these higher-order complexes.

Table I. Solubility (μg/ml) at 25°C of I-III in Water, 25% HPB, and 25% HPG

	I	II	III
H_2O	2.42	12.5*	4.95*
25% HPB	2,850	1,270	650
25% HPG	290	270	110

* Single determination.

The molecular interaction between I and HPB was further characterized by the thermodynamic parameters associated with the inclusion complex formation. As shown in Figure 2, temperature significantly affected the interaction. For 1:1 complex formation, the equilibrium constant for the complexation K_{eq} was determined according to:

$$K_{eq} = \frac{m}{S_0(1 - m)}$$

where m is the initial slope shown in Figure 2 and S_0 is the intrinsic solubility (8). In turn, this allowed to calculate $\Delta G^o = -RT\ln K_{eq}$. We also estimated ΔH^o directly from the van't Hoff equation assuming temperature-independent ΔC_p (plot not shown). Finally, ΔS^o was obtained from $(\Delta H^o - \Delta G^o)/T$. Table II contains the summary of the intrinsic solubility of I at various temperatures and estimates of thermodynamic parameters.

As shown in Table II, K_{eq} varies from 3.38×10^4 M^{-1} at 47°C to 4.53×10^4 M^{-1} at 4°C. In magnitude, they are comparable with the association constant for the interaction between phenol and β-cyclodextrin (21). The signs of all thermodynamic parameters associated with the complex formation between I and HPB (Table II) indicate spontaneity of the association. The large positive ΔS^o observed (~13 eu) indicates a typical hydrophobic interaction between HPB and I, as has been also reported for the interaction between L-phenylalanine and α-cyclodextrin (21). It seems reasonable that the major molecular force involved in the interaction between I and HPB is the hydrophobic interaction between the benzyl group at C7 and the cavity of HPB filled with water molecules. In this context, it is interesting to note that the most water-insoluble compound I resulted in the greatest increase in solubility with the cyclodextrin derivatives. This observation is in agreement with other studies (22).

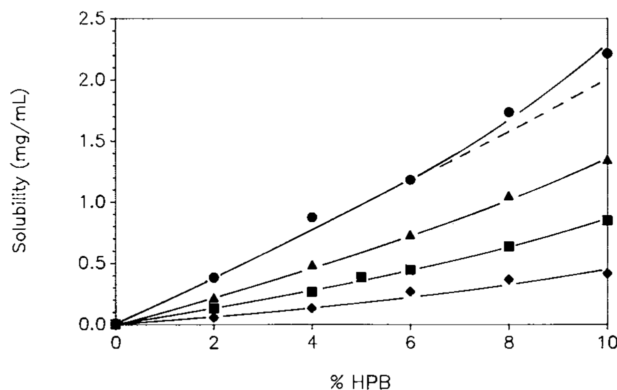


Fig. 2. Apparent solubility of I in HPB solutions at 45°C (circles), 37°C (triangles), 25°C (squares), and 4°C (diamonds).

Table II. Intrinsic Solubility (S_o) of Compound I and Thermodynamic Parameters Associated with the Molecular Interaction Between I and HPB

Temp (°C)	S_o (μg/ml)	S_o (μM)	K_{eq} (M^{-1})	$\Delta H°$ (kcal/mole)	$\Delta G°$ (kcal/mole)	$\Delta S°$ (eu)
4	1.03	2.93	4,530		−4.64	12.6
25	2.42	6.89	4,000	−1.14	−4.91	12.7
37	4.42	12.6	3,850		−5.09	12.7
47	9.73	27.7	3,380		−5.17	12.7

Transepithelial Transport of I in the Presence of HPB

The effect of HPB on the transport of I across the cell monolayer can be determined using a series of stock solution in which either the concentration of HPB varies at a constant concentration of I or the latter varies at a constant HPB concentration. Each of these protocol can be followed with or without continuous rocking of the cell monolayer system in A-to-B or B-to-A transport direction. In the present study, we chose to monitor the transport in A-to-B direction using two test solutions in which the total concentration of I was held constant while HPB concentration was either 2 or 10%. The initial concentration of I should be high enough that at each time interval, either 60 or 120 min, its concentration in the B side can be determined with confidence. As shown in Figures 1 and 2, one can prepare a highly concentrated solution of I by simply employing a high HPB concentration. In such a situation, however, we confronted two problems. First, the fractional concentration of unbound I varies only in infinitesimal quantities; for instance, 0.01 to 0.1%. Secondly, it was noticed that, at a total concentration of I greater than approximately 0.25 mg/ml, the cell monolayer tends to lose its viability/integrity. The latter was determined by [^{125}I]-BSA leakage from the B side, [^{14}C]HPB or [^{14}C]sucrose leakage from the A side, and microscopic examination of morphology at the end of an experiment after glutaraldehyde fixation and hematoxylin/eosin staining (15). Similarly a suspension of I at solubility equilibrium without any HPB was found to be cytotoxic. Transport experiment with I alone in a test solution was impossible due to the low intrinsic solubility. Therefore, we chose test solutions of total concentration 0.152 mg/ml (4.33 × 10^{-4} M) of I in the transport studies. The results of the transport experiments are shown in Figure 3 and Table III.

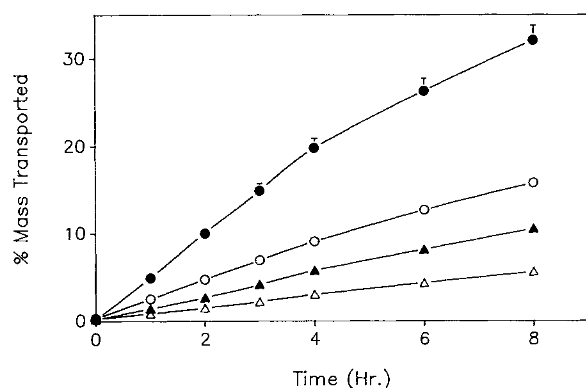


Fig. 3. Apical-to-basolateral transport of I across the MDCK cell monolayer of 4.71 cm^2 at 37°C with (closed symbols) or without (open symbols) continuous rocking of the system. In all cases, the initial concentration I was constant at 0.152 mg/ml while HPB concentration was either 2% (circles) or 10% (triangles). Viscosity of the 2% HPB solution containing I was adjusted to that of 10% HPB solution at 37°C with Ficoll® 400. Data points are average of quadruplicate determinations and one SD is shown as a bar. The apical donor sides of all 16 cell monolayers were also spiked with [^{14}C]HPB at the beginning of the experiment, however, no significant radioactivity was detected in the basolateral side in any time during the 8-hour transport study.

In the presence of HPB, there are two possible species of I that can undergo transport across the cell monolayer; the unbound drug or the complex with HPB. However, 99.7 ± 2.0% (n = 16) of the radioactivity from [^{14}C]HPB added to the apical donor side was recovered from the same side and no significant radioactivity from [^{14}C]HPB was detected in the basolateral side after the 8-hr transport experiment. Therefore, the possibility that I passes through the call monolayer as a complex is ruled out. The complex must have dissociated prior to the transport. Two possible mechanisms are proposed to explain the uptake of unbound I by the cell. First, a sequential dissociation-diffusion-partition process can provide free drug molecules to the cell. Here, a possibility that dissociation of the complex might be rate-limiting is unlikely, for the kinetics of inclusion complexation is generally instantaneous (14). Alternatively, the complex can transfer the drug molecule directly to the apical domain of the cell membrane via a collision-mediated process. In both mechanisms desorption from the basal domain to the albumin-containing medium in the receiving compartment cannot be rate-limiting, for I was not accumulated in the cell layer; the total recovery was invariably in the range of 95 to 105% (also see 16). Partition coefficient of I between n-oc-

Table III. Initial Transport Rate of I at 37°C Across the MDCK Cell Monolayer under Dynamic or Static Condition in the Presence of 2 or 10% HPB in Donor Compartment: Initial [I] = 152 μg/ml (4.33 × 10^{-4} M), and the fractional concentration of unbound I is calculated according to the K_{eq} reported in Table II (also see Fig. 3).

Total HPB (%)	Total HPB (10^2 × M)	Free I (%)	Free I (μg/ml)	Flux (10^4 × μg/cm^2 · sec)	
2	1.30	2.0	3.08	D*	6.67
2	1.30	2.0	3.08	S	3.14
10	6.49	0.4	0.61	D	1.86
10	6.49	0.4	0.61	S	1.00

* D and S represent the flux under dynamic and static conditions, respectively.

tanol/H_2O was estimated to be 2.4×10^4 (CLOGP; Daylight CIS, Inc., Irvine, CA). For such a lipophilic compound, membrane partition is more favorable than diffusion across the unstirred aqueous boundary layer. The latter being rate-limiting is supported by the finding that with gentle agitation, the overall transport increased approximately 2-fold.

The effective diffusivity of I in 2 or 10% HPB solution should be nearly identical, since the complex is the predominating species in both solutions; the fractional concentration of the 1:1 complex are 98.0 and 99.6% in 2 and 10% HPB solutions, respectively. This is in spite of the approximation based on the Stoke-Einstein equation (23) that the diffusion coefficient of the complex should be 1.75-fold greater than that of free I molecule. Therefore, had the collision-mediated process dominated the transport of I, an identical initial influx is expected under a given hydrodynamic condition. On the other hand, if the unbound drug is exclusively transported through the sequential dissociation-diffusion-partition process, a 5-fold increase in flux is expected as the HPB concentration decreases from 10% to 2% which resulted in a 5-fold increase in the unbound drug concentration under a given hydrodynamic condition. However, we observed 3.1- and 3.6-fold increases under the static and dynamic condition, respectively. This analysis implied a combined contribution from both mechanisms to the overall flux. Based on the significant difference in flux observed with difference in free drug concentration, it is speculated that the sequential dissociation-diffusion-partition process plays a more important role than the collision-mediated process.

Taken together, the data presented in this communication emphasize the importance of the fractional concentration of the free drug, which is the major species diffusing across the aqueous boundary layer, in the overall transport across a cell monolayer. The GI absorption of water-insoluble lipophilic substances can be significantly enhanced by optimizing the concentration of complexing ligand such that the free drug concentration approaches its intrinsic solubility.

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