Flutamide - Hydroxypropyl-ß-cyclodextrin Complex: Formulation, Physical Characterization, and Absorption Studies using the Caco-2 in vitro Model

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ABSTRACT The objective of this research was to formulate flutamide (FLT) in hydroxypropyl-ß-cyclodextrin (HPßCyD), and to investigate FLT transcellular permeation from the complex using the Caco-2 monolayer *in vitro* model. **Methods:** Classical solubility data were used to derive thermodynamic parameters which, together with Differential Scanning Calorimetry (DSC), ^{1}H-NMR and ^{19}F-NMR, were used to characterize and derive stability constants for the FLT-HPßCyD complex. The Caco-2 cell line was used to examine the role of HPßCyD on the passage of FLT across cell monolayers *in vitro*. **Results:** The solubility of FLT in water (1.46 mmol/L) increased almost 170 times (to 243.45 mmol/L) in the presence of 50% (w/v) HPßCyD. Solubility data for FLT in aqueous HPβCyD were used to derive thermodynamic parameters ($\Delta G°$ at 298 K = -3.48, $\Delta H°$ = 2.85, $\Delta S°$ at 298 K = 21.24). The solubility of FLT in HPßCyD increased proportionally with an increase in temperature. The FLT-HPßCyD complex had an A_L-type (DSC) isotherm, consistent with a linear increase in FLT solubility and unchanged stoichiometry. The DSC of free FLT and HPßCyD showed endothermic peaks at 110 °C and 300 °C, respectively. FLT-HPßCyD did not display a free-FLT endothermic response, but exhibited broadening of the endothermic peak in the HPßCyD region. ^{19}F- and ^{1}H-NMR chemical shifts moved upfield as a function of its increased solubility in the presence of HPßCyD. The FLT-HPßCyD stability constant, K_S (1:1) was estimated to be 356 M^{-1} and 357 M^{-1}, from thermodynamic and ^{19}F NMR data, respectively. The apical-to-basal permeability coefficient (P_{eff} = 4.75×10^{-5} $cm.s^{-1}$) for FLT across Caco-2 cell monolayers at 37 °C increased as HPβCyD concentrations were reduced, indicative of transepithelial passage via passive diffusion of available free FLT in solution. Studies in the presence and absence of Ca^{2+} ruled out a significant paracellular transport component. **Conclusions:** FLT-HPßCyD is a relatively stable, 1:1 inclusion complex. Formation of this complex substantially increases the water solubility of FLT, but HPßCyD, except in high dilution, reduces transcellular passage of FLT in the Caco-2 cell *in vitro* model.

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INTRODUCTION

Flutamide (2-methyl-N-[4-nitro-3-(trifluormethyl)-phenyl] propanamide; FLT; Fig. 1) is a non-steroidal fluorine-containing antiandrogen used in prostate cancer chemotherapy (1). FLT is a prodrug that is

rapidly metabolized to hydroxyflutamide, its major, active metabolite (2). The recommended dose (250 mg orally three times daily) is associated with nausea, diarrhea, vomiting and increased appetite (3-5). The low water solubility (40 µg/mL) and poor wettability of FLT may contribute to its low absorption from the commercially available tablets, and preclude intravenous (*iv*) loading. Formulations that produce higher concentrations of FLT in solution may therefore provide important therapeutic options for patients.

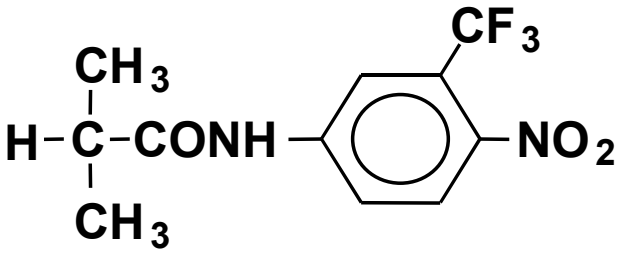

Figure 1. Chemical structure of flutamide (FLT).

Cyclodextrins (CyDs) are "host" molecules that trap a great variety of "guest" molecules having the size of one or two benzene rings, and thereby increase their water solubility without the need for organic co-solvents or surfactants. Beta cyclodextrin (βCyD; 7 glucose residues) has ideal dimensions to complex a range of commonly used drugs. Unfortunately, it also has a particularly high affinity for cholesterol, forming a poorly soluble cholesterol-βCyD complex which may crystallize in the kidneys and cause nephrotoxicity. Hydroxypropyl-ß-cyclodextrin (HPßCyD), a chemical derivative of ßCyD, similarly improves the solubility of many drugs, but it is more hydrophilic than the ßCyD, forms a less stable complex with cholesterol, and is therefore less toxic. (6). This work therefore focuses on increasing the solubility of FLT by forming an FLT-HPßCyD inclusion complex.

The inadequate absorption of FLT through the gastrointestinal tract (GI) may not be due to its inability to permeate the intestinal epithelium, but to its low concentration at the absorption surface. The most commonly used, commercially available, human intestinal cell line for drug absorption studies is Caco-2 (7-9). The Caco-2 cell line differentiates spontaneously to enterocyte-like cells under conventional cell culture conditions. Advantages of the Caco-2 cell line model include rapid evaluation of the transepithelial permeability coefficients (P_{eff}) and absorption mechanisms under controlled conditions.

The objectives of this study were to solubilize FLT in the presence of HPβCyD, to physicochemically characterize the FLT-HPβCyD inclusion complex, and to study the passage of FLT across Caco-2 monolayers.

MATERIALS AND METHODS

FLT, HPßCyD (average molar substitution 0.8), polyethylene glycol ($M_n \cong 200$), dimethylsulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), L-glutamine, transferrin, penicillin-streptomycin, insulin, trypsin and EGTA (ethylene glycol-*bis*-(β-aminoethyl ether)-*N,N,N',N'*–tetraacetic acid) were purchased from Aldrich Chemical Co. PBS (pH 7.4) was prepared in-house (contains 140 mM NaCl, 260 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4). PBS^+ used in the transport experiment was prepared by adding 225 µL of 2 M $CaCl_2$ and 200 µL of 1M $MgCl_2$ to 500 mL of normal PBS. All chemicals and solvents were commercial analytical reagent grade; HPLC grade distilled water was used throughout the study.

FLT was analyzed spectrophotometrically at 300 nm in methanol:water (1:1 v/v) (PHILIPS PU 8740 UV/VIS Scanning Spectrophotometer). The measured ε_{300nm} was 5709 mol^{-1} cm^{-1}.

Solubility. Excess FLT was added to aqueous solutions of HPβCyD (0 to 333.3 mM) in 20-mL screw-capped vials. After 2 min. of vortex mixing (Vortex Genie^{TM}) and 5 min. of sonication in a water bath (Branson 2200), samples were shaken in a water bath (Dubnoff Metabolic Shaking Incubator) at 25°C for 3 h. Preliminary experiments showed that 3 h was an appropriate equilibration time. Approximately 0.5 mL of the solution was withdrawn by 1 cc syringe and filtered through a 0.45 µm membrane (Millipore)

fixed in a holder (Nucleopore). These phase solubility experiments were repeated at temperatures at 30, 37, 45, 50 °C. Filtrate (0.20 mL) was withdrawn from each sample at each temperature and analyzed by UV after appropriate dilution with methanol: water (1:1 v/v). These solubility data were used to calculate the binding constants for formation of the FLT-HPßCyD complex. The Van't Hoff equation was used to derive the thermodynamic parameters. (10).

FLT-HPßCyD inclusion complex formation. Excess FLT was added to an aqueous solution of HPßCyD (50% w/v). This mixture was filtered and the filtrate was lyophilized (Freeze Dryer 3, Labconco) to produce the solid inclusion complex as a white powder.

Differential Scanning Calorimetry (DSC). A DSC 120 (SEIKO SII, model SSC/5200) differential scanning calorimeter was used. Free FLT, HPßCyD, the physical mixture of FLT and HPßCyD, and the inclusion complex (FLT-HPßCyD) were sealed in separate aluminum pans, with a sealed empty aluminum pan as reference. The transition temperature of each sample was obtained from its DSC plot.

Nuclear Magnetic Resonance Spectroscopy. ^{19}F NMR spectra were recorded at 270 MHz on a Bruker AM 300 spectrometer equipped with MacSpect 3 data module. Solutions of FLT (0.5 mM) and HPßCyD (1.7 mM to 33.3 mM) were prepared in D_2O with KF (0.2 M) as internal standard. ^{19}F chemical shifts were compared with a reference sample that contained only FLT and KF in water. Samples containing excess FLT in D_2O, or excess FLT in 1% (w/v) HPßCyD in D_2O, were equilibrated (2 min vortex mixing, 5 min sonication and 3 h shaking in water bath) and filtered for ^1H NMR analysis at 500 MHz on a Varian spectrometer.

Caco-2 cells. Caco-2 cells were maintained at 37 ^oC in DMEM containing 20% (v/v) fetal bovine serum, 0.058% (w/v) glutamine, 0.001% (w/v) transferrin, 1% (v/v) PBS, and 50 unit/100 mL insulin, in a 5% CO_2, 90 % relative humidity atmosphere. Cells grown in 25 cm^2 flasks (Corning Costar) and passaged every 5 days at a split ratio of 1:5 reached confluence within 6-7 days after passage. For FLT transport studies, 1.4×10^6 cells (Spencer hemocytometer) were seeded in each Transwell® insert (24.5 mm diameter, 4.71 cm^2, pore size 0.4 µm, Corning Costar). The medium in the Transwell® was changed daily. The integrity and permeability of cell monolayers was determined by transepithelial electrical resistance (TEER) measurements (MILLICELL®ERS, ENDOHM-24). PBS^+ (1 mL) was added to the apical chamber of the Transwell and 4 mL of PBS^+ were added to the basolateral chamber, respectively. TEER values across the Transwell filter, in the absence of cells, were used as reference.

Caco-2 trans-monolayer passage of FLT. Passage of FLT from the apical to the basolateral chamber was studied when the TEER of cell monolayers on the Transwell reached 200 Ω cm^2. FLT or FLT-HPβCD solutions (various concentrations in 1.5 mL of PBS^+ or medium) were carefully added onto the apical surface of the monolayer. During incubation at 37 °C, FLT concentrations in the apical and basolateral chambers were periodically measured by HPLC (Waters 501 HPLC pump, Waters 486 Tunable Absorbance Detector, Waters Radial Pak C18 10 µm reverse-phase column and µBondapak™ C18 Guard-Pak™ guard column). To study the role of paracellular passage of FLT, the Caco-2 cell monolayer was pretreated for 45 min with a low calcium medium containing 2.5 mM EGTA to complex free Ca^{2+} ions. Sink conditions were maintained in the basolateral chamber by moving the Transwell® to a fresh PBS^+ well of a 6-well plate at predetermined intervals. The integrity of the monolayers was checked by measuring TEER at the end of each experiment.

Data analysis. The effective permeability coefficient (Peff) was calculated using the equation (11):

$$P_{eff} = V/A * C_0 \, x \, dc/dt$$

where dc/dt, the flux across the monolayer (FLT, mM/s), is the initial slope of a plot of the cumulative

receiver concentration (basolateral chamber) versus time, V is the volume of the receiver chamber (mL), A is the surface area of the monolayer (here 4.7 cm^2) and C_0 is the initial concentration (mM FLT) in apical compartment.

RESULTS AND DISCUSSION

Solubility of FLT at different concentrations of HPβCyD. The solubility of FLT increased approximately 170-fold in the presence of HPβCyD (Table 1). The solubility limit of the complex was not reached within the range of concentrations of HPβCyD used in this study. The stoichiometry and solution stability of the inclusion complex can be determined from the slope and intercept of the phase-solubility plot of FLT solubility as a function of HPβCyD concentration. The DSC isotherm is type A_L, implying a linear increase in solubility with unchanged stoichiometry and a 1:1 FLT:HPβCyD binding ratio (12). This complex is highly water-soluble at room temperature, since no precipitation was observed even at HPβCyD concentrations as high as 0.35 M. The solubility of FLT increased almost 170 times at the highest HPβCyD concentration used. The stability constant K_S for 1:1 FLT-HPβCyD was calculated to be 356 M^{-1}, using the equation:

$$K_S\ (1:1) = Slope / S_O(1-Slope)$$

where S_O is the solubility of the FLT in the absence of HPβCyD (13).

Table 1. Thermodynamic parameters for complexation of FLT with HPβCD (n = 1).

Stability Constants (M^{-1})					ΔG°(298K) kcal.mol^{-1}	ΔS°(298K) cal.K^{-1}.mol^{-1}	ΔH° kcal.mol^{-1}
323K	318K	310K	303K	298K			
452	444	386	341	318	-3.48	21.24	2.85

Temperature effect on the aqueous solubility of FLT in HPβCyD. The solubility of FLT in 5% HPβCyD increased proportionally with increases in temperature. Thermodynamic parameters calculated from these data are shown in Table 2. FLT dissolution thermodynamics in aqueous HPβCyD were characterized by a negative ΔG°, indicative of spontaneous dissolution; and a positive ΔH°, indicative of endothermic dissolution. It is reported that the driving forces for inclusion complexation between CyD and a guest molecule may include Van der Waals interactions, hydrogen bonding, hydrophobic interactions, release of high-energy water molecules from the cavity of CyD, and release of strain energy in the ring of CyD (14). Usually complex formation with HPβCyD results in a relatively large negative ΔH°, and a ΔS° that can be either positive or negative (15). The large ΔS° (+20 cal.K^{-1} $mole^{-1}$) for FLT can be attributed to the transfer of FLT from aqueous medium to a more apolar site, such as the cavity of HPβCyD. This transfer involves breakdown of water structure around FLT, which creates a large positive ΔS° and a small positive ΔH°, apparently governed by hydrophobic interactions.

Table 2. The solubility of FLT in aqueous solutions of HPβCyD (0 to 333 mM) at 25° C.

HPβCyD Concentration		Flutamide Solubility
mmol/L	w/v %	mmol/L
0	0	0.146
13.3	2	0.898
33.3	5	2.022
66.7	10	4.025
133.3	20	7.967
200.0	30	13.195
266.7	40	18.338
333.3	50	24.345

DSC analysis. The DSC isotherms of free FLT and HPβCyD are characterized by sharp endothermic peaks at 110 °C and 300 °C, respectively, and a

physical mixture of FLT and HPßCyD exhibits both of these endothermic peaks, although the peak for HPßCyD is only barely discernable. However, the DSC of the FLT-HPßCyD complex showed no endothermic peak for FLT, and the endothermic peak of HPßCyD was appreciably broadened (Figure 2). This was taken as another indication of inclusion complex formation.

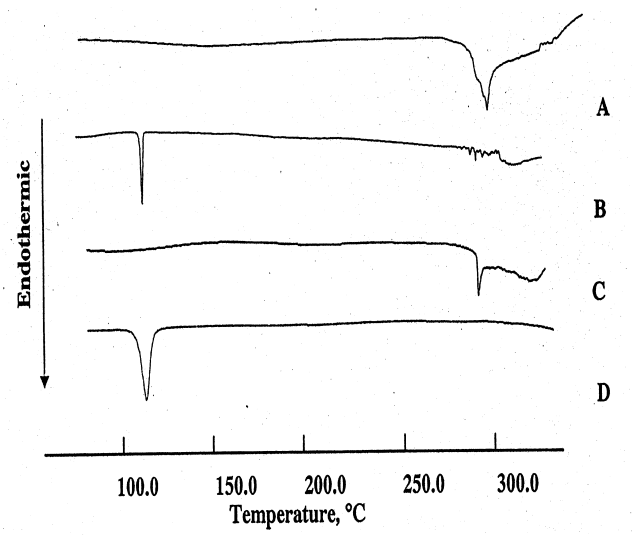

Figure 2. DSC spectra for (A) the FLT-HPβCyD inclusion complex, (B) a physical mixture of FLT and HPβCyD, (C) HPβCyD, and (D) FLT.

NMR studies. DSC is only one of the analytical methods used to confirm the formation of inclusion complexes. Although the DSC test was positive for FLT complexation with HPßCyD, NMR was used for further confirmation. The ^{19}F NMR chemical shifts of fluorine atoms in FLT, as a function of the concentration of HPßCyD, are shown in Table 3. The observed maximum chemical shift change reached 1.36 ppm, in 33.3 mM HPβCyD. This was taken as further proof of the formation of an inclusion complex between HPβCyD and FLT. The free and complexed forms of FLT gave rise to only one NMR fluorine signal, possibly due to fast exchange between free and bound FLT at equilibrium. The stability constant (K_S) for a 1:1 complex in the presence of a large excess of HPβCyD, was derived from NMR parameters using the Benesi-Hildebrand method (16, 17):

$$\Delta\delta_{obs} \cdot ([HP\beta CyD]_{total})^{-1} = K_S \cdot (\Delta\delta_c - \Delta\delta_{obs})$$

where $\Delta\delta_{obs}$ is the ^{19}F chemical shift difference between free FLT (in the absence of HPβCyD) and the observed value from each sample. $[HP\beta CyD]_{total}$ refers to the total concentration of HPβCyD, including free and complexed HPβCyD. $\Delta\delta_c$ represents the ^{19}F chemical shift difference between free FLT and FLT in the pure HPβCyD-complex. The negative slope of a plot $\Delta\delta_{obs} \cdot ([HP\beta CyD]_{total})^{-1}$ against $\Delta\delta_{obs}$ will generate K_S. The K_S obtained from this procedure is 357 M^{-1} at 298 K, and is in good agreement with the value obtained from the phase solubility test (356 M^{-1} at 298 K). Since the CF_3 group is on the aromatic ring, and because the F atoms are undergoing a change in chemical shift, one may rationalize that the benzene ring enters the cavity of HPβCyD.

Table 3. Effect of HPβCD on the ^{19}F NMR chemical shift of FLT (n = 1).

$\Delta\delta_{obs}$ (ppm)	[HPβCD] (mM)	^{19}F NMR chemical shift (ppm)
0.00	0.0	59.75
0.52	1.7	60.27
0.81	3.3	60.56
1.01	10.0	60.76
1.18	13.0	60.93
1.36	33.3	61.11

The HPβCD used in the project was a mixture of substitution isomers with an average molar substitution of 0.8. Although the ^1H chemical shift changes of such a mixture of HPβCD's was too complex for analysis, the readily observed chemical shift changes of the FLT aromatic protons further support the model in which the aromatic ring is inserted into the hydrophobic inner cavity of HPβCD. The FLT proton resonances in D_2O occur at 4267,

4000 and 4105 Hz. Decoupling the ^{1}H doublet at 4000 Hz (aromatic region) of the proton NMR spectrum collapsed it to a singlet at 4105 Hz. In the FLT-HPβCyD complex, the singlet at 4267 Hz shifted upfield to 4242 Hz, while the doublet at 4000 Hz shifted downfield to 4084 Hz. The shift of the doublet at 4105 remained unchanged.

Passage of FLT across Caco-2 cell monolayers. Studies of FLT passage across Caco-2 monolayers at several concentrations of HPβCyD (0.15 to 2.5%) revealed that both the percent passing through the monolayer and the P_{eff} decreased with the increasing concentrations of HPβCyD (Figure 3). These data imply that the P_{eff} increases when more free FLT is available, and that trans-monolayer passage is due to passive diffusion of available free FLT. Since dilution will increase FLT release from its HPβCyD inclusion complex, it will increase the diffusion of FLT across the monolayer. There are no literature reports of FLT metabolism by Caco-2 cells, and HPLC analysis for FLT in the apical and basolateral chambers during these studies did not detect any evidence of metabolites.

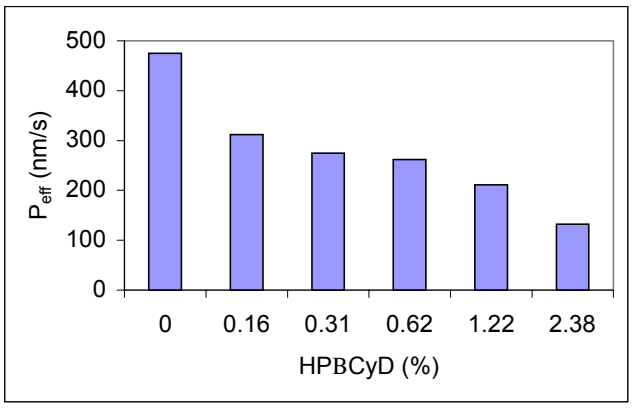

Figure 3. The effective permeability coefficient (P_{eff}) of FLT at several concentrations (% w/v) of HPβCD in PBS.

Haeberlin *et al*. (18) reported on CyD-associated absorption enhancement. They found that β-, γ-cyclodextrin, HPβCyD and DMβCyD did alter oral absorption of modified calcitonin and octreotide *in vitro* in Caco-2 cell monolayers and *in situ* in isolated rat jejunal sections, by enhancing permeation of the mucosal membrane. Hovgaard and Brøndsted (19) studied the effects of CyD's as absorption enhancers using the Caco-2 monolayer model. β-CyD (1.8 %), α- and γ-CyD (5 %) and HPβCD (5 %) were compared with DMβCyD (2.5% and 5%). They concluded that DMβCyD was the most effective in increasing the permeability of the cytoplasmic membrane in a concentration dependent manner. No significant beneficial effects on transport were seen for the other CyDs relative to the control.

Drugs that are well orally absorbed *in vivo* have threshold P_{eff} values around 10^{-6} cm/s in the Caco-2 cell line, while drugs that have lower P_{eff} values are poorly absorbed in animals or humans (20). By extension, current experiments indicate that FLT (P_{eff} = 4.75×10^{-5} cm.s^{-1}) should be well absorbed. If this is correct, then its low bioavailability may be due to the low amount of free drug in solution, which in turn reflects slow dissolution from the formulation.

Epithelial cells are joined through a complex of three separate structures: tight junctions, intermediate junctions and desmosomes. The integrity of these structures, and consequently the paracellular passage of drugs, is dependent on Ca^{+2} ion concentration (21). The contribution of paracellular pathways to the overall trans-monolayer passage of FLT was investigated by replacing the PBS in the standard the low Ca^{2+} buffer cell culture medium [DMEM] with normal PBS^{+} buffer containing 0.9 mM Ca^{2+}. This increases the Ca^{2+} concentration to at least 10 times higher than the normal intracellular concentration (the 100 nM range) (22). After a 45 min FLT absorption experiment with EGTA (2.5 mM) in the buffer to chelate Ca^{2+}, the TEER returned to normal values within a 2 h recovery time, indicating that membrane changes due to calcium removal were reversible. It has been reported that exposure of the cells to low Ca^{2+} medium for a time longer than 90 min will result in an irreversible decrease in the TEER (23). In the current experiments, reversible opening of the monolayer tight junctions had no apparent effect on the trans-monolayer movement of FLT (Figure 4). This implies that the trans-cellular passage of FLT is the major contributing pathway,

and that the paracellular pathway is of limited importance for FLT absorption.

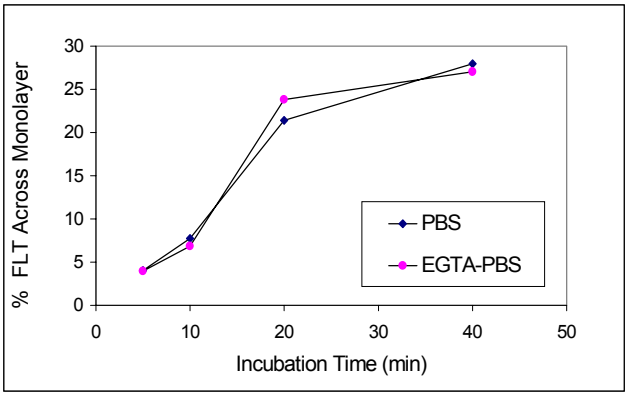

Figure 4. The trans-membrane passage of FLT under conditions in which tight junctions are either open (-♦-) or closed (- ● -).

The Caco-2 monolayers have TEER > 200Ω·cm^2, high enough to reduce the transport of hydrophilic compounds to a very low level, so that any small change in paracellular permeability is readily detectable. In general, the opening of the tight junction complex has no effect on the passage of the more lipophilic drugs. These drugs partition rapidly into cell membranes, and their distribution to the intercellular spaces will therefore be limited even after EGTA treatment, since the cell membranes cover a much larger surface area than the intercellular spaces. However, more hydrophilic drugs have lower solubility in the cell membranes, and trans-membrane passage via paracellular channels will be more important for them.

SUMMARY AND CONCLUSIONS

The water solubility of FLT was increased 170-fold in the presence of 50% (w/v) HPßCyD. Thermodynamic parameters derived from FLT solubility in the presence of various concentrations of HPßCyD at several temperatures revealed that the solubility of FLT increased proportionally with an increase in temperature. FLT-HPßCyD inclusion complex formation was characteristic of a very strong hydrophobic interaction. The complexes were characterized by an A_L-type DSC isotherm indicative of a linear increase in FLT solubility with unchanged stoichiometry of the complex. DSC of free FLT and HPßCyD displayed endothermic peaks at 110 °C and around 300 °C, respectively, whereas the DSC of the complex gave no indication of free FLT.

^{19}F NMR chemical shifts of F atoms in FLT-HPßCyD moved downfield as a function of increased FLT solubility in the presence of HPßCyD. The stability constants calculated based on the ^{19}F NMR chemical shift were similar to one calculated from the phase solubility test. ^{1}H NMR further confirmed the inclusion formation, showing aromatic proton chemical shift changes upon the inclusion complex formation.

The apical-to-basal passage of FLT across Caco-2 cell monolayers at 37 °C was transcellular, with little or no evidence for paracellular passage. Apical-to-basal passage decreased with increasing HPβCD concentrations, indicating that transcellular passage is due to passive diffusion of free drug reaching the cell surface.

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