Transport and uptake characteristics of a new derivative of berberine (CPU-86017) by human intestinal epithelial cell line: Caco-2

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KEY WORDS CPU-86017; berberine; Caco-2 cell; cyclosporine A; sodium dodecyl sulfate; sodium citrate; sodium deoxycholate

ABSTRACT

AIM: The characteristics of transepithelial transport and uptake of CPU-86017 {[7-(4-chlorbenzyl)-7,8,13,13α-tetrahydroberberine chloride, CTHB]}, a new antiarrhythmia agent and a new derivative of berberine, were investigated on epithelial cell line (Caco-2) to further understand the absorption mechanism of berberine and its derivatives. **METHODS:** Caco-2 cell was used. **RESULTS**: 1) The permeability coefficient from the apical (AP) to basolateral (BL) of CPU-86017 was approximately 5 times higher than that from BL-to-AP transport. The effects of a P-glycoprotein (P-gp) inhibitor-cyclosporin A, some surfactants, and lower pH on the transepithelial transport of CPU-86017 were also observed. Cyclosporine A at 7.5 mg/L had no effect on the transepithelial electrical resistance (TEER); an about 4-fold enhancement on the transepithlial transport of CPU-86017 was observed. Some surfactants (sodium citrate, sodium deoxycholate, and sodium dodecyl sulfate) at 100 μmol/L and low pH (pH=6.0) induced a reversible decrease of TEER; enhancements of the transepithelial transport of CPU-86017 were also observed with some surfactants; 2) In the process of uptake of CPU-86017, the initial uptake rates of CPU-86017 were saturable with a V_{max} of (250±39) μg·min^{-1}·g^{-1} (protein) and K_m of (0.90±0.12) mmol/L. This process was enhanced by cyclosporine A (7.5 mg/L) with a V_{max} of (588±49) μg·min^{-1}·g^{-1} (protein) and K_m (0.42±0.08) mmol/L. **CONCLUSION**: Some surfactants and P-gp inhibitors can be considered as enhancers of its transepithelial transport and uptake.

INTRODUCTION

The human Caco-2 cell line has been used as an *in vitro* model to study the absorption of many drugs[1]. This model system consists of monolayers of a highly differentiated human colon adenocarcinoma cell line grown onto microporous polycarbonate membranes. Under the culture condition, these cells possess many biochemical and morphological properties of the intestinal epithelial cells[2]. Cells become polarized after confluence under culture condition and possess microvilli on the apical surface (facing the growth medium) and a smooth surface on the basolateral (support) side.

CPU-86017, [7-(4-chlorbenzyl)-7,8,13,13α-

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tetrahydroberberine chloride, CTHB] (Fig 1), a berberine derivative, is a newly developed complex class III antiarrhythmic agent[3]. CPU-86017 had the effects of antiarrhythmia[3] and reduced the cardiac muscle hypertrophy induced by *L*-thyroxin[4]. CPU-86017 is poorly absorbed in animal experiments. The absolute bioavailability of CPU-86017 is only 10 %-12 % in dogs[5]. In our study, the transepithelial transport and uptake characteristics of CPU-86017 were assessed in the Caco-2 cell model. The effects of surfactants, cyclosporine A, and lower pH on the transepithelial transport and uptake characteristics of CPU-86017 were also studied.

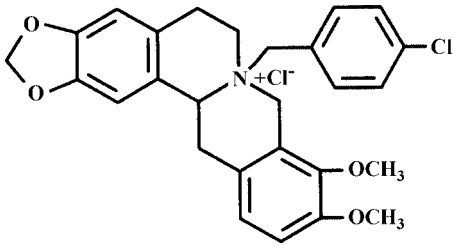

Fig 1. The structure of CPU-86017 ($C_{27}H_{27}CL_2NO_4$). M_r=500.43 ; purity 99.0 %.

MATERIALS AND METHODS

Materials The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). High glucose Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco company (Paisley, UK). Six-well Transwells™ were from Corning Costar Corporation (Cambridge, MA, USA). Fetal bovine serum (FBS) was from TDB Company (Nanjing, China). Non-essential amino acids (NEAA), *L*-glutamine, benzylpenicillin, and streptomycin were from Kewei Company (Nanjing, China). Epithelial Voltohmeter (EVOM) was purchased from World Precision Instrument (New Haven, CT, USA). Cyclosporine A and CPU-86017 (purity >99 %) were obtained from the New Drug Research Centre in China Pharmaceutical University. [^{14}C]mannitol was from NEN Company (Boston, Mass. USA). All other chemicals were reagent grade. HPLC, fluorescent detective and Shimpack ODS column (5 μm, 4.6 mm×10 cm) were from Shimadzu Company (Shanghai, China).

Transepithelial transport Caco-2 cells were planted at a density of 62 000 cells/cm^2 on the Transwell™ polycarbonate membranes coated with rat-tail collagen I. The culture medium consisted of high glucose DMEM, 10 % FBS, 1 % NEAA, benzylpenicillin 100 kU/L, and streptomycin 100 mg/L. After confluence (about 5-6 d), the culture medium was replaced daily (1.5 mL topside and 2.6 mL basolateral side). Cells were cultured 21 d for using. Cells were incubated at 37 ºC, in an atmosphere of 5 % CO_2 and 90 % relative humidity. All cells were used in this study between passages 27-40. The integrity of the cell monolayer was checked by measuring the transcellular flux of [^{14}C]mannitol and the transepithelial electrical resistance (TEER) across the monolayers. The normal TEER values range from 470-600 Ω·cm^2, and the normal [^{14}C]mannitol flux was less than 0.2 % h^{-1}·cm^2.

Prior to transport studies, Caco-2 cell monolayers were equilibrated with Hanks' balanced salt solution (pH 7.2, HBSS) for 30 min. The topside of the cell monolayers is defined as the apical side (AP, 1.5 mL) and the surface facing a microporous polycarbonate membrane defined as the basolateral side (BL, 2.6 mL). CPU-86017 was added into donor side. Samples 50 μL were taken from receiver side at 0,15, 30, 45, and 60 min. The same volume of HBSS was added to keep the volume of receiver side after each sampling. Permeability coefficient (P_{app}) is obtained by dividing the unidirectional fluxes by the drug concentration in the donor solution:

$$P_{app} = \frac{\Delta Q}{\Delta t \cdot A \cdot C_0} \quad (1)$$

P_{app} is apparent permeability, ΔQ is the amount of drug transported in Δt, A is the area of Transwell™, C_0 is the initial concentration of drug.

For the dilution effect caused by sampling, the cumulative amount transported is given by

$$TR_{cum} = A_n + \frac{Vs_n}{V_R}\sum_{i=0}^{n-1} A_i \quad (2)$$

Where A_n is the amount of drug measured in sample n. V_{sn} is the volume of sample, and V_R is the volume of the receiver side.

The permeability of CPU-86017 from AP to BL side was determined when different chemicals were present in HBSS during incubation period. These chemicals are cyclosporine A (7.5 mg/L), sodium citrate 100 μmol/L, sodium dodecyl sulfate 100 μmol/L, and sodium desoxycholate 100 μmol/L.

Uptake kinetics of CPU-86017 on Caco-2 cell monolayers Caco-2 cells were plated in six-well plates

for 21 d for uptake experiment.

Effect of incubation time Having been washed with HBSS at 37 ºC three times, the cells were incubated with CPU-86017 10 mg/L in 2 mL HBSS at 37 ºC for 0, 5, 10, 15, and 20 min. The cell monolayers were washed three times with ice-cold HBSS after incubation. Then, cell monolayers were removed into 1.0 mL saline and homogenized by supersonics. The amount of CPU-86017 was then analyzed by HPLC. The amount of protein in the cell homogenates was measured by the method of Bradford[6]. Uptake is expressed as μg/g.

Effect of pH Having been washed with HBSS at 37 ºC three times, the cells were incubated with CPU-86017 10 mg/L in 2 mL HBSS with pH 6.0, 7.0, and 8.0 separately at 37 ºC for 10 min. HCl and $NaCO_4$ were used to adjust the pH of HBSS. Then, cell monolayers were treated with the same way as above.

Uptake kinetics of CPU-86017 on Caco-2 cell monolayer and effects of cyclosporine A Having been washed with HBSS at 37 ºC three times, the cells were incubated with: (1) CPU-86017 0, 0.05, 0.2, 0.5, 1.0, and 5 mmol/L at 37 ºC or 4 ºC for 10 min; (2) CPU-86017 0, 0.05, 0.2, 0.5,1.0, and 5 mmol/L with cyclosporin A (7.5 mg/L) at 37 ºC for 10 min. The cell monolayers were washed three times with ice-cold HBSS after incubation. Then, cell monolayers were treated with the same way as above. And the uptake at 4 ºC was subtracted from the uptake obtained at 37 ºC to reduce the affects of temperature.

Determination of CPU-86017 CPU-86017 was determinated by HPLC with fluorescence detection. The column is Hypersil ODS 4.6 mm×10 cm, and mobile phase is MeOH: Acetonitrile=65:35, including 0.1 % triethylamine. The excitation wavelength is 210 nm and emission wavelength is 320 nm. The column temperature is 20 ºC. The flow speed of mobile phase is 1.0 mL/min. Injected volume of sample is 20 μL.

Statistics Data were expressed as mean±SD. The *t*-test was used for statistical analysis and statistical significance was defined as $P<0.05$ or $P<0.01$.

RESULTS

Linearity, precision, and accuracy of CPU-86017 determination Under the condition described above, the retention time of CPU-86017 is 4.1 min (Fig 2).

Accuracy and precision for the assay were determinated with CPU-86017 at 5, 50, and 500 μg/L (each in six replicates).

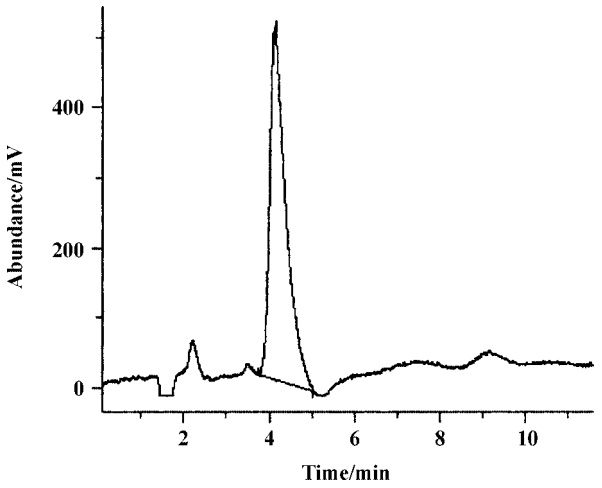

Fig 2. Representative chromatograms of CPU-86017 in Hanks' balanced salt solution (HBSS).

Calibration curves were plotted as the peak area *vs* drug concentration. Results for the calibration curve ($n=6$) showed good linearity ($r=0.997$) over the concentration range of 5.0-500.0 μg/L, with an equation of $y=29612x-49051$ (y=area; x=concentration in mg/L).

Accuracy and precision for the assay were determined by calculating the intra-day and inter-day variation at three concentrations of 5.0, 50.0, and 500.0 μg/L in six replicates. The intra-day RSD was less than 10 %; the inter-day RSD was less than 20 % (data not shown). These results indicated that the method was reliable within the analytical ranges.

Transport of CPU-86017 across Caco-2 monolayers The results of the transepithelial transport of CPU-86017 from AP-to-BL and BL-to-AP were presented in Fig 3. At d 21, the P_{app} in AP-to-BL was about 5 times less than that in BL-to-AP (Tab 1).

In the AP-to-BL transport of CPU-86017, cyclosporine A 7.5 mg/L, sodium citrate 100 μmol/L, sodium deoxycholate 100 μmol/L can greatly increase the P_{app} of CPU-86017 (Fig 4, 5, and Tab 1). A reversible decrease of TEER in this process was observed when the surfactants were added to HBSS. When these surfactants were removed, TEER returned to the normal levels in 8 h after experiments (data not shown).

In the lower pH (pH=6, compared with pH 7.2) and with dodecyl sodium sulfate, the value of P_{app} had some increase, but it did not have significant difference in statistic.

Uptake of CPU-86017 into Caco-2 monolayers The uptake of CPU-86017 was time-, pH-, concentra-

Tab 1. Comparison of permeability coefficient (P_{app}) in different conditions. *n*=5. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

$10^7 \times P_{app}$/cm·s^{-1}						
AP-BL (Control)	BL-AP	AP-BL Cyclosporine A 7.5 mg/L	AP-BL Sodium citrate 100 μmol/L	AP-BL SDS 100 μmol/L	AP-BL Sodium deoxycholate 100 μmol/L	AP-BL pH=6
5.0±1.3	27±7^{c}	19±4^{c}	8.8±2.9^{b}	5.9±1.9	11.8±1.3^{c}	6.7±1.5

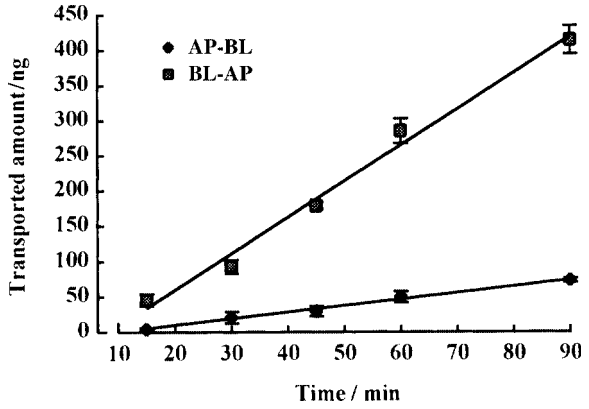

Fig 3. Transepithelial transport of CPU-86017 across Caco-2 cell monolayers grown on Transwell™ (21-d old). Samples were withdrawn from the receiver side at the times indicated and analyzed by HPLC. *n*=5. Mean±SD.

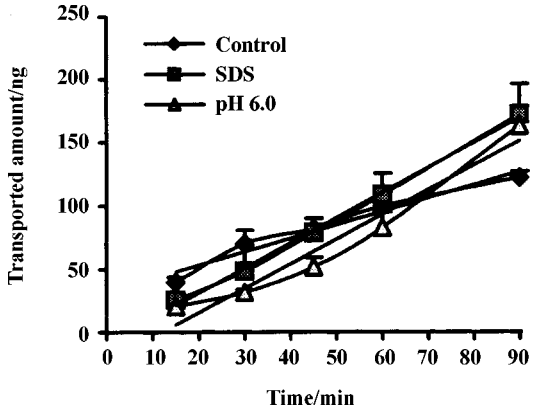

Fig 5. Effects of sodium dodecylsulfate (SDS 100 μmol/L) and pH on the transport of CPU-86017 (AP-to-BL). Samples were taken from the receiver side at the times indicated and were analyzed by HPLC. *n*=5. Mean±SD.

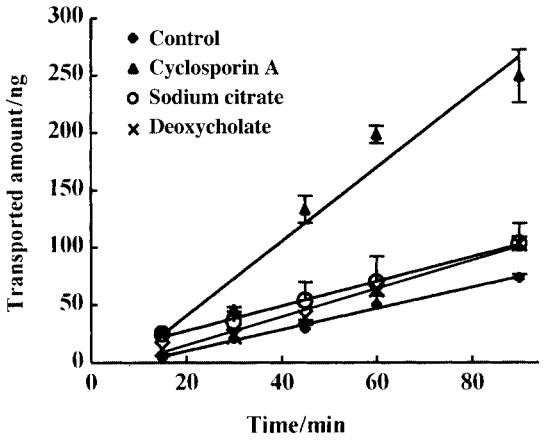

Fig 4. Effects of different materials on the transport of CPU-86017 (AP-to-BL). Samples were taken from BL side at the times indicated and were analyzed by HPLC. *n*=5. Mean±SD.

tion-, and temperature-dependent.

Effect of time and pH on the uptake of CPU-86017 The uptake of CPU-86017 into Caco-2 monolayers was shown to be linear with respect to time from 0 to 20 min at 37 °C (Fig 6). The uptake of CPU-86017 into Caco-2 monolayers decreased with increase of pH from 6.0 to 8.0 (Fig 7).

Effect of concentration and temperature on the uptake of CPU-86017 The flux rate constants

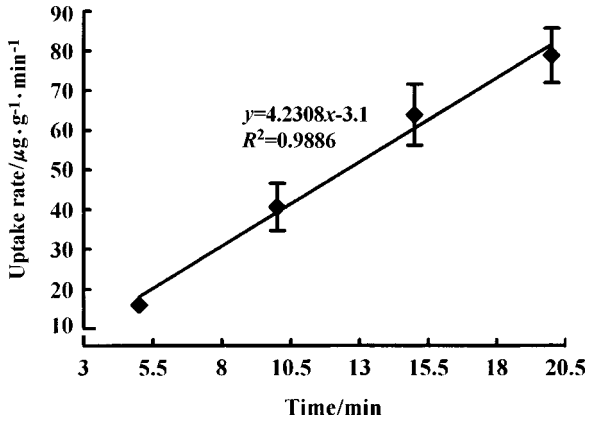

Fig 6. Time dependence of CPU-86017 uptake. Caco-2 cells were incubated with CPU-86017 10 mg/L at 37 °C for 0-20 min. The line represents a liner regression with R^2>0.99. *n*=5. Mean±SD.

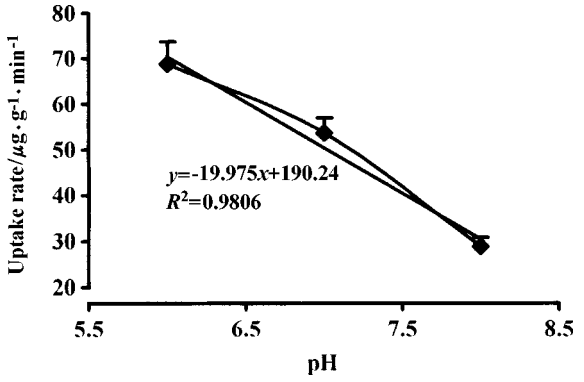

Fig 7. Effects of pH on the uptake of CPU-86017. Uptake experiments with CPU-86017 10 mg/L were performed at 37 °C for 10 min. The line represents a liner regression with R^2>0.95. n=5. Mean±SD.

for the uptake of CPU-86017 at 37 °C decreased with increasing concentrations of CPU-86017 (Fig 8). The effects of temperature was reduced by subtracting the uptake obtained at 4 °C from that obtained at 37 °C (Fig 9). The results indicated that the uptake of CPU-86017 could be explained by Michaelis-Menten formulation but not only by simple diffusion. Linear least-squares regression analysis on the double-reciprocal plot of the rate of uptake *vs* concentration data provided a good correlation (R^2=0.992). The slope (k_m/V_{max}) and intercept ($1/V_{max}$) of the regression equation were 3.6×10^{-3} and 4×10^{-3} respectively. The uptake parameters of V_{max} and k_m were (250±39) μg·min^{-1}·g^{-1} (protein), and (0.90±0.12) mmol/L, respectively.

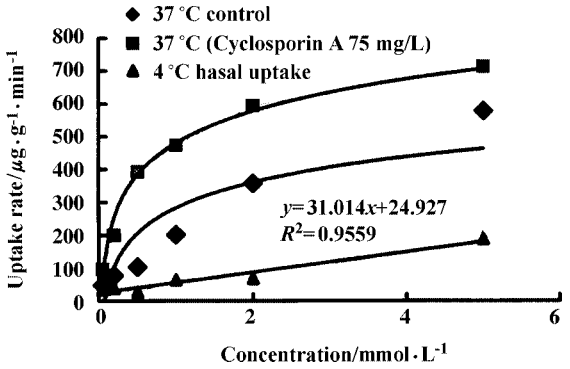

Fig 8. Concentration-dependence of CPU-86017 uptake. The cell homogenates were analyzed by HPLC. The uptake at 4 °C was subtracted from the uptake obtained at 37 °C. n=5.

After given cyclosporine A, the uptake procedure was enhanced, the slope (k_m/V_{max}) and intercept ($1/V_{max}$) of the regression equation were 7×10^{-4} and

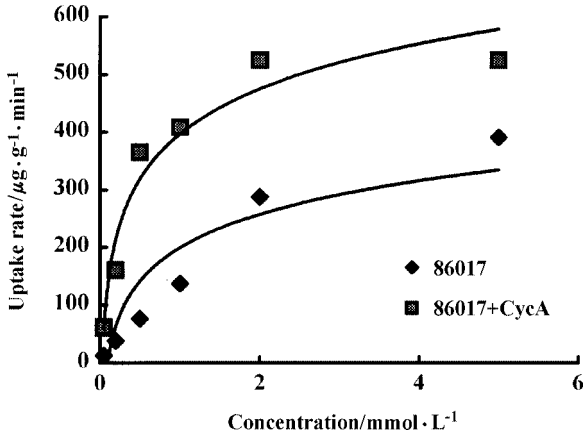

Fig 9. Concentration-dependence of CPU-86017 uptake after subtracting the uptake at 4 °C, under control conditions and in the presence of cyclosporin A (7.5 mg/L).

1×10^{-3} respectively. The uptake parameters of V_{max} and k_m were (588±49) μg·min^{-1}·g^{-1} (protein) and (0.42±0.08) mmol/L, respectively (Fig 10).

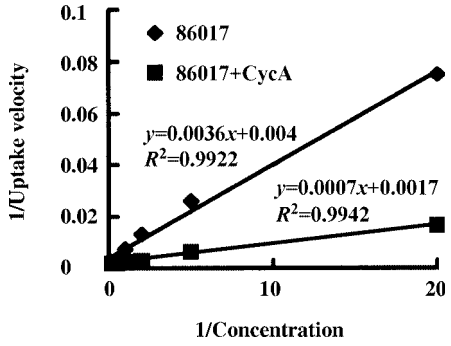

Fig 10. Reciprocal curve of CPU-86017 uptake velocity (μg·g^{-1}·min^{-1}) and concentration (mmol/L) after temperature correction, under control conditions and in the presence of cyclosporin A (7.5 mg/L).

DISCUSSION

Caco-2 cell monolayers could achieve functional polarity and integration under cultured condition[7]. In this study, Caco-2 cell monolayers were cultured for 21 d. The value of TEER reached to a stable level. The leakage of [^{14}C]mannitol did not change significantly in monolayers older than 18 d, showing the stability of the barrier properties of the monolayers in long times (results not shown).

Transport of CPU-86017 The fact that CPU-86017 was transported much faster from the BL to the AP side than in the opposite direction is indicative of substantial polarity of the transport system. The BL-

to-AP polarity was strongly positive.

Berberine and its derivatives are regarded as substrates of P-glycoprotein. They can up-regulate the expression of the 170 kDa P-glycoprotein and their transepithelial transport can be enhanced by P-gp inhibitors[12,13]. In CPU-86017 transport experiment, the AP-to-BL P_{app} of CPU-86017 increased significantly when cyclosporine A (7.5 mg/L) was added into the system. So P-glycoprotein can also inhibit the AP-to-BL transepithelial transport of CPU-86017.

In the transepithelial transport of CPU-86017, sodium deoxycholate, sodium dodecyl sulfate, and sodium citrate greatly increased the AP-to-BL P_{app} of CPU-86017.

It is considered that some surfactants can loosen the Ca^{2+}-dependent occluding junction, and then increase the transport of many drugs[10]. Also, many literatures suggested that sodium deoxycholate enhanced drug transepithelial transport by different mechanisms, including: (a) it can build a polarity channel by forming reverse micellar, this channel permits polar molecules to diffuse through plasmalemma[11]; (b) it can reduce the viscosity of the mucus on the surface of epithelia, and make it easy for drugs to permeate though plasmalemma[12]; (c) it can interact with Ca^{2+}, affecting the formation of tight junction and thus increasing the permeation through paracellular pathway[9]; (d) it can reverse the effect of multi-drug resistance[14]. In the transport of CPU-86017, the concentration of sodium deoxycholate is only 100 μmol/L , which is far less than 5 mmol/L, the critical micelle concentration (CMC)[11]. So, sodium deoxycholate may enhance the transport of CPU-86017 by loosening the tight junction of Caco-2 cell monolayers in the experiment or by reversing the effect of P-gp.

In the transepithelial transport process, reversible decreases of TEER were observed when surfactants exist in the system. So, it seems that they affect the tight junctions of Caco-2 cell monolayers, then enlarging the intercellular space and increasing the transport of CPU-86017.

Uptake of CPU-86017 In the uptake experiments, the flux rate constants for the uptake of CPU-86017 were shown to decrease with the increase of CPU-86017 concentration. The relationship between uptake rate and concentration of CPU-86017 fitted procedure of Michaelis-Menten formulation. The uptake parameters of V_{max} and K_m were (250±39) μg·min^{-1}·g^{-1} (protein) and (0.90±0.12) mmol/L respectively.

The results also indicated that P-glycoprotein played a role in this process. Cyclosporin A can facilitate the uptake procedure and increase the V_{max} from (250±39) μg·min^{-1}·g^{-1} (protein) to (588±49) μg·min^{-1}·g^{-1} (protein).

In addition to dependency on the concentration, the effects of time, pH and temperature on the uptake of CPU-86017 in Caco-2 monolayers were also studied in this research. For example, the uptake of CPU-86017 (10 mg/L) was reduced dramatically when the temperature was reduced from 37 ºC to 4 ºC. The uptake of CPU-86017 at 4 ºC is in accord to first-order velocity procedure. Its slop is 32.6 mg·min^{-1}·mmol^{-1}·g^{-1} (protein). The uptake of CPU-86017 increased from 28.4 to 67.6 mg/g (protein) in Caco-2 monolayers when pH reduced from 8.0 to 6.0.

The results of transport and uptake of CPU-86017 indicate that the poor absorption of CPU-86017 is determined by its intrinsic permeability. P-glycoprotein may be the other reason for the poor absorption of CPU-86017, for CPU-86017 is one of the substrates of P-glycoprotein. From the experiments, it was considered that some substances such as sodium citrate and sodium deoxycholate could lead to the promotion of the absorption of CPU-86017. So, adding surfactants or P-gp inhibitors seemed to be a promising approach to increase the transepithelial transport and uptake of CPU-86017.

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