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Research paper

# Influence of cyclodextrins and chitosan, separately or in combination, on glyburide solubility and permeability

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**Abstract**

The effect of chitosan and of different concentrations of β- or hydroxypropyl-β-cyclodextrins, separately or in various (w/w) combinations, on the dissolution characteristics of glyburide (an oral hypoglycemic agent subject to incomplete and variable bioavailability) and on its permeability through Caco-2 cells has been investigated. Cyclodextrins (and particularly the hydroxypropyl-derivative, in virtue of its higher water solubility) were clearly more effective than chitosan in enhancing the drug dissolution properties: the aqueous glyburide solubility was improved 40-fold in the presence of 25 mM hydroxypropyl-β-cyclodextrin, 25-fold in the presence of 13 mM β-cyclodextrin (saturation solubility) and only 3-fold in the presence of chitosan at its saturation concentration (0.5% w/v). When chitosan and cyclodextrin were simultaneously present, a strong reduction of the cyclodextrin solubilizing efficiency towards the drug was observed, and it was attributed to a possible competition effect of polymer and glyburide for the interaction with the macrocycle. By contrast, permeation studies revealed that chitosan was more powerful than cyclodextrins in enhancing the glyburide permeability through Caco-2 cells. This was probably in virtue of the polymer's favourable effect on the tight junctions opening, as demonstrated by the significant decrease in the transepithelial electrical resistance recorded in its presence. Moreover, interestingly, when using the carriers together, conversely from solubility studies, a significant ($P<0.05$) synergistic effect in enhancing glyburide apparent permeability was revealed in permeation experiments.


*Keywords:* Glyburide; Solubility; Permeability; Caco-2 cells; Cyclodextrins; Chitosan

## 1. Introduction

Glyburide is an oral hypoglycemic agent belonging to the second generation of sulfonylureas commonly employed in the treatment of type II non insulin-dependent diabetes. However, its very low water solubility can cause incomplete and variable bioavailability and, consequently, possible bio-inequivalence among its pharmaceutical dosage forms [1–3]. Over the last few years, various approaches aimed at enhancing glyburide dissolution properties have been made, such as drug amorphization [4], solid dispersion [5–8] or complexation with cyclodextrins [9,10].

Chitosan [(1->4)-2-amino-2-deoxy-β-D-glucan] is a linear cationic natural polysaccharide which presents several desirable biological properties such as non-toxicity, high biocompatibility and biodegradability. In recent years it has been extensively investigated for its potential in the development of several drug delivery systems, due to its bio-adhesiveness and transmucosal penetration enhancer abilities, accompanied by wide availability in nature, low cost and high flexibility in use [11–13]. Furthermore, it has demonstrated its effectiveness in improving dissolution properties and bioavailability of poorly-soluble drugs [14–17].

On the other hand, the solubilizing capacities of cyclodextrins towards a number of drugs are well known [18,19]. In addition, their possible enhancer effect on drug permeation through cell membranes has been reported [20–23] and different mechanisms have been proposed by the various authors for explaining this effect.

Furthermore, the possible use of cyclodextrins as candidates for coenhancer effect could be considered. In fact, it has been shown that the presence of cyclodextrins may promote the potency of other absorption enhancers [24,25]. However, the negative effects on drug bioavailability of the combined use of

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cyclodextrins and some hydrophilic polymers has been also observed [26].

Therefore, in the present work we considered it worthy of interest to evaluate the effect of cyclodextrins and chitosan, either separately or in different (w/w) combinations, on both the solubility and permeability characteristics of glyburide. Caco-2 cells, a colon rectal adenocarcinoma cell line of human origin, were selected as the in vitro model for permeability studies, since they have shown to be a valuable tool to investigate human intestinal absorption of drugs [27–29].

## 2. Materials and methods

### 2.1. Materials

Glyburide (GB) was obtained from Guidotti Laboratori S.p.A. (Pisa, Italy). Hydroxypropyl-β-Cyclodextrin MS 0.9 (HPβCd) and β-Cyclodextrin (βCd) were a kind gift from Roquette Italia S.p.A. Chitosan (CS) (molecular weight 150, 000, deacetylation degree 75–85%) was supplied by Sigma Chem. Co. (St Louis, USA). Solvents used in the HPLC procedure were of HPLC grade. All other reagents were of analytical grade.

### 2.2. Solubility studies

Solubility studies were performed by adding an excess of drug (70 mg) to 15 mL of water (pH ≈ 6.3) or unbuffered aqueous solutions of CS (from 0.025 to 0.5% w/v) (pH ≈ 6.5), or Cd (from 5 to 25 mM) (pH ≈ 6.2) in sealed glass vials electromagnetically stirred (500 rpm) at constant temperature (25 °C) until equilibrium (2 d). An aliquot of solution was then withdrawn with a filter-syringe (pore size 0.45 μm), and the drug concentration was spectrometrically determined at 300 nm (UV/Vis 1601 Shimadzu). The presence of Cd and/or CS did not interfere with the assay. Each experiment was performed in triplicate (coefficient of variation C.V. $<2.5\%$).

### 2.3. Cell cultures

The Caco-2 cell line was kindly provided by Dr A. Zweibaum and Dr M. Rousset (INSERM U170, Villejuif, France). Cells were grown at 37 °C in a 10% $CO_2$/90% air atmosphere using as culture medium a Dulbecco's modified Eagle's solution (pH 7.4) additioned of 10% fetal bovine serum (FBS) and 1% non-essential amino-acids (NEAA) (Life Technologies Eragny, France). Cells at passages 80–85 were seeded at a density of $10^2$ cells/cm$^2$ on polycarbonate filters (area 1.13 cm$^2$) in Costar Snapwell six-well plates (Costar Europe Ltd, Badhoevedorp, The Netherlands). The culture medium was added with 110 IU/mL benzylpenicillin G and 100 μg/mL streptomycin sulphate (Life Technologies Eragny, France) and changed every second day. Cell cultures were kept at 37 °C in an atmosphere of 95% relative humidity, 10% $CO_2$. Filters were used for transport studies 21–28 days after seeding [29].

### 2.4. Transport studies

Test solutions were made up of pH 7.0 buffered Hanks' balanced salt solution (HBSS) containing $4.0\times10^{-2}$ mM GB (drug saturation solubility) alone or in the presence of CS (0.0625% w/v) and/or Cd (βCd 3.75 or 7.5 mM or HPβCd 7.5, 15 or 25 mM). For the dynamic transport experiments, filters were placed into Grass-Swettana chambers and pre-incubated at 37 °C, in the presence of 1.5 mL pH 7.0 buffered HBSS on both the sides of the chambers in an atmosphere of 95% air and 5% $CO_2$. After equilibration, the medium at the apical (AP) side of the cells was replaced by 3.0 mL of test solution (donor compartment). At predetermined times 20 μL samples, withdrawn from the basolateral (BL) side (i.e. the acceptor compartment) and replaced with an equal volume of fresh HBSS solution, were assayed for drug content by HPLC as described below (Section 2.6). Results were corrected for dilution and expressed as cumulative transport as a function of time. Each experiment was performed on six filters contemporaneously.

The apparent permeability coefficient was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{1}{A}\frac{1}{C_0}$$

where $P_{app}$ is the apparent permeability coefficient (cm/s), $dQ/dt$ (μg/s) the rate of appearance of the drug on the basolateral side, $A$ the surface area of the monolayers (1.13 cm$^2$), and $C_0$ (μg/mL) the initial drug concentration in the donor compartment.

In the case of drug alone, additional permeation trials were carried out at 4 °C with the aim of evidencing possible mechanisms of active transport.

A mass balance calculation was always performed to determine if phenomena of accumulation, metabolism of the solute or adsorption to the apparatus occurred [30].

The results of all the permeation experiments, expressed as average values ± SD ($n=6$), were statistically analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls multiple comparison post test (Graph Pad Prism, Version 3). The differences were regarded as statistically significant when $P<0.05$.

### 2.5. Measurement of the transepithelial electrical resistance (TEER)

Measurement of TEER was performed to evaluate possible damage of the cellular monolayer during the experiments. The TEER values were calculated by determining the potential difference between the two faces of the cell monolayer using a Millicell® ERS meter (Millipore, Bedford, MA, USA) connected to a pair of chopstick electrodes [31]. Measurements started 10 min prior to incubation on the apical side of the cells with the testing and blank solutions. All experiments were performed in sextuple in a 95% air and 5% $CO_2$ atmosphere at

37 °C. Average TEER values for untreated cell monolayers were over 350 $\Omega \times cm^2$.

Fluorescent hydrophilic Lucifer Yellow (St Quentin Fallavier, France) was employed as marker to check the junction integrity of the Caco-2 cell monolayer during the experiments. 300 μL of a HBSS solution of Lucifer Yellow (3 mg/100 mL) were added to the apical side and, at time intervals, samples withdrawn from the basolateral side were assayed using a fluorescence 96-wells plate reader Cytofluor™ 4000 (Perkin–Elmer). Results were corrected for dilution and expressed as cumulative transport as a function of time.

### 2.6. High performance liquid chromatography (HPLC) assay

HPLC analyses were performed with a Shimadzu SPD-6A apparatus equipped with an injector valve with a 20 μL sample loop (Mod. Rheodyne) using a C18 Hypersil ODS (5 μm 250 × 4.6 mm) column. The mobile phase consisted of a mixture of acetonitrile/water (60:40 v/v). The flow rate was 1.0 mL/min (LC-10AS Shimadzu pump). GB was detected spectrometrically (UV–vis SPD-10A Shimadzu) at 230 nm. No interference was found for the marker Lucifer Yellow.

### 2.7. Lactate dehydrogenase (LDH) cytotoxicity text

LDH test was used to evaluate the cytotoxicity of the tested carriers. LDH is a stable cytoplasmic enzyme normally present in most cells; it is released into the cell culture supernatant upon damage of cellular membrane. LDH test allows determination of LDH release from cells based on a colorimetric quantitation after an enzymatic reaction [32,33]. LDH leakage from Caco-2 cells was measured by using the assay-kit provided by Roche diagnostics (Meylan, France). Briefly, after incubation in the wells, the cells were washed with pH 7.0 buffered HBSS and then additioned of 200 μL of medium containing the product to be tested. Spontaneously released LDH in intact cells (low control) and maximum LDH released after cell lysis using 1% v/v of Triton® X-100 solution (high control) were also determined. The plates were incubated for 120 min at 37 °C. After incubation, 100 μL of supernatant were transferred from each well to a new plate and added of 100 μL of reconstituted reaction medium. The wells were incubated at 37 °C for 30 min, and then the enzymatic reaction was stopped by adding 50 μL/well of 1 N HCl solution. Absorbance values were measured within 1 h at 490 nm with the ELISA reader. Cytotoxicity of the samples was calculated as follows:

$$\%\text{cytotoxicity} = \frac{A_{\text{sample}} - A_{\text{spont}}}{A_{\text{max}} - A_{\text{spont}}} 100$$

where $A_{\text{sample}}$ is the absorbance value for the cells treated with the sample, $A_{\text{spont}}$ the value for the spontaneous LDH release and $A_{\text{max}}$ the value in lysed cells in the presence of Triton®.

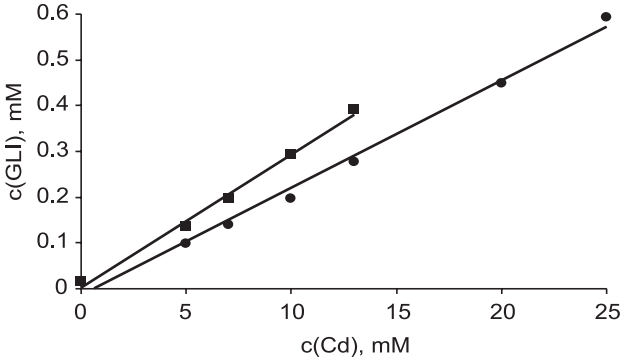


Fig. 1. Phase-solubility diagram of glyburide (GB) in the presence of increasing concentrations of HPβCd (●) or βCd (■).

## 3. Results and discussion

### 3.1. Phase-solubility studies

The aqueous solubility of GB linearly increased with increasing the concentration of both native and hydroxypropylated βCd, giving in all cases $A_L$ type phase-solubility diagrams [34] (Fig. 1). The apparent 1:1 binding constants of the GB–Cd complexes, calculated from the slope of the straight lines of the phase-solubility diagrams [34] were $1873 \pm 60\ M^{-1}$ for βCd and $1497 \pm 45\ M^{-1}$ for HPβCd. The slight decrease of the complex stability constant observed for the βCd-derivative, with respect to that of the parent one, suggested that the presence of hydroxypropyl substituents could hinder the inclusion of the drug into the Cd cavity owing to the partial obstruction of its opening. An analogous phenomenon was previously observed for other drugs such as ibuprofen [35] or ibuproxam [36]. On the other hand, our result disagreed with that of Savolainen et al. who found a reversed trend for the binding constants of complexes of HPβCd and βCd with GB [10]. A possible explanation for these contrasting results could be the very different Cd concentration range used in phase-solubility studies (0–25 or 0–75 mM, respectively). This same consideration is also true for the possible formation of GB–Cd complexes of 1:2 mol/mol stoichiometry, observed in the presence of high ligand concentrations [10]. However, in virtue of its higher water solubility, the solubilizing efficiency of HPβCd, calculated as the ratio between GB solubilities in 25 mM HPβCd aqueous solution and in pure water, was about 40, clearly greater than that of the parent compound at its water saturation concentration (13 mM), which was 25. Also the presence of CS in the aqueous solution improved the drug's water solubility (Fig. 2), but the solubilizing efficiency of the polymer, calculated at its highest concentration used (0.5% w/v, i.e. the polymer aqueous saturation solubility), was only about three.

Unexpectedly, when phase-solubility experiments were performed with Cds in the presence of 0.0625% w/v CS (i.e. the polymer concentration used in permeation studies), the drug solubility improvement was clearly lower than in the presence of Cd alone, indicating a possible competition effect

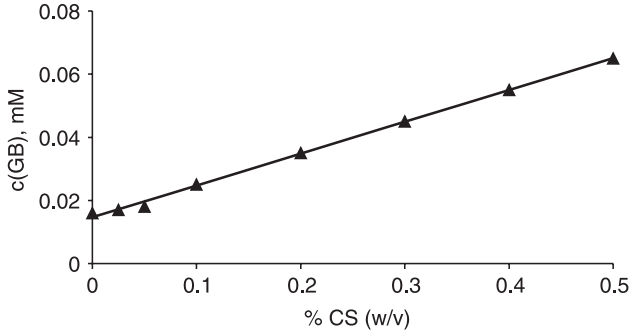


Fig. 2. Phase-solubility diagram of glyburide (GB) in the presence of increasing concentrations of chitosan (CS) (▲).

between polymer and drug for the interaction with the macrocycle (Fig. 3). In particular, the solubilizing efficiency of both Cds decreased by about four to five times passing from 40 to 8 for HPβCd (at 25 mM conc) and from 25 to 6 for βCd (at 13 mM conc) and the apparent stability constants of the related drug-Cd complexes dropped to 295 and 395 $M^{-1}$, respectively.

### 3.2. Transport studies

Subsequently, in order to evaluate if the examined carriers (used both separately and in combination) also have some effect on the GB permeability, we performed a series of transport studies across Caco-2 cells. This cell line was selected, since it has shown to be a valuable model for the evaluation of absorption enhancers, and, in particular, it has been successfully used for studying the possible effect of CS [11,29,31,37,38] or Cds [39–42] on drug absorption promotion. Different Cd concentrations were tested in drug transport studies, in the presence or not of a fixed concentration of CS (0.0625% w/v). This latter was selected, on the basis of the LDH test, as the highest CS concentration with acceptable cytotoxicity, i.e. about 25%. This limit was selected considering that values up to 20% toxicity (determined by LDH test) corresponded to about 100% of cell survival determined by measurements of mitochondrial dehydrogenase activity (MTT test) [43]. Neither βCd nor HPβCd were cytotoxic to Caco-2 cells, according to the LDH test, even at the highest concentration used (13 and 25 mM, respectively).

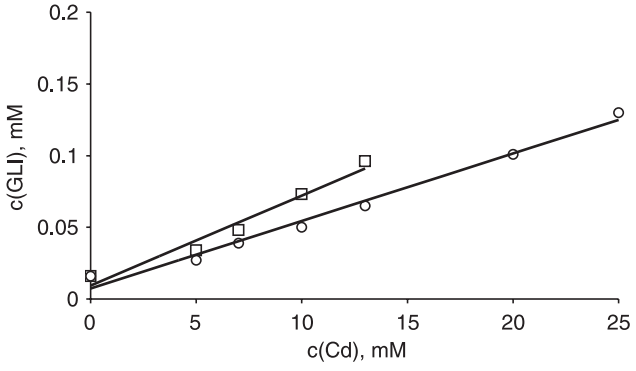


Fig. 3. Phase-solubility diagram of glyburide (GB) in the presence of increasing concentrations of HPCβd (○) or βCd (□) and a fixed concentration (0.0625% w/v) of chitosan (CS).

Table 1
Apparent permeability ($P_{app}$) values across Caco-2 cells at 37 °C of glyburide (GB) alone or in the presence of 0.0625% w/v chitosan (CS) or different βCd or HPβCd concentrations, with or without 0.0625% w/v CS (standard deviations in brackets)

| Sample | $P_{app}$ $10^{-5}$ (cm/s)[a] |
|---|---|
| GB alone | 2.16 (0.25) |
| GB + CS | 3.4 (0.65)[b] |
| GB + 3.75 mM βCd | 2.1 (0.28) |
| GB + 7.5 mM βCd | 2.50 (0.40) |
| GB + 3.75 mM βCd + CS | 3.30 (0.52)[b,c] |
| GB + 7.5 mM βCd + CS | 4.15 (0.57)[b,c,d] |
| GB + 7.5 mM HPβCd | 2.15 (0.26) |
| GB + 15 mM HPβCd | 2.38 (0.44) |
| GB + 25 mM HPβCd | 2.60 (0.57) |
| GB + 7.5 mM HPβCd + CS | 3.24 (0.42)[b,c] |
| GB + 15 mM HPβCd + CS | 4.20 (0.67)[b,c,d] |
| GB + 25 mM HPβCd + CS | 4.79 (0.93)[b,c,d] |

[a] Mean ± SD ($n = 6$).
[b] Significantly different ($P < 0.05$) from GB alone.
[c] Significantly different ($P < 0.05$) from the corresponding sample without CS.
[d] Significantly different ($P < 0.05$) from sample with CS alone.

In all cases, the transport of the drug was linear over the time periods studied. The AP-to-BL apparent permeability values ($P_{app}$) of GB alone or in the presence of the different carriers examined, separately or in combination, calculated from permeation profiles at 37 °C, are shown in Table 1.

The AP-to-BL $P_{app}$ value of GB alone determined at 4 °C was $1.97 \times 10^{-5}$ cm $s^{-1}$, i.e. very similar to the value obtained at 37 °C ($2.16 \times 10^{-5}$ cm $s^{-1}$), indicating that mechanisms of active transport were not involved in the GB cellular membrane permeation process.

As can be seen from the data in Table 1, the permeation enhancer power towards the drug of the two types of carriers (CS and Cd) was in the opposite order than that observed for their solubilizing efficacy. In fact CS, while used at a very low concentration (0.0625% w/v) in order to avoid any problem of cellular toxicity, was clearly effective as absorption enhancer, giving an about 60% increase of GB apparent permeability. No significant $P_{app}$ variation ($P > 0.05$) of GB with respect to the drug alone was instead found at any of the tested concentrations of both βCd or HPβCd.

It is generally recognised that Cds act as carriers by keeping hydrophobic drug molecules in solution and delivering them to the surface of biological membranes where they partition into the membrane. Therefore, if the carrier simply acts as a solubility enhancer and does not interfere with cell membrane integrity and permeability (since drug permeation occurs by passive diffusion of free drug reaching the cell surface), the drug flux will increase with Cd concentration when the drug is in suspension, and decrease when Cd is in excess [20,44]. Thus, under the present experimental conditions (drug saturation solubility), we should observe a reduction of drug permeation in the presence of Cd, and such an effect should become more evident with increasing the Cd concentration. On the contrary,

we found a slight increase, although not statistically significant ($P > 0.05$), of the drug $P_{app}$ value. As a possible explanation of such findings, it could be hypothesized that Cd has some permeation enhancer effect towards GB, but it is almost counterbalanced by the reduction of free drug available for absorption, due to the complex formation.

Very interesting results were obtained when CS and Cd were used in combination. In fact, in spite of the negative effect observed in solubility studies, the simultaneous presence of the two types of carriers showed, by contrast, a favorable synergistic effect in improving drug permeability. The drug $P_{app}$ values obtained when CS and Cd were present together were significantly higher ($P < 0.05$) not only than those obtained with all the corresponding samples with Cd alone, but also, over a threshold concentration of Cd, than those with CS alone (Table 1). Moreover, it can be observed that the threshold concentration was lower for βCd (7.5 mM) than for HPβCd (15 mM). However, the greater water solubility of the hydroxypropyl-derivative allowed use of higher concentrations in its combinations with CS (up to 25 mM), thus obtaining higher drug $P_{app}$ values, up to an about 120% $P_{app}$ increase in comparison with the drug alone.

In accordance with the previous formulated hypothesis, these results could be explained by considering that, as found in phase-solubility studies, CS strongly reduces the GB–Cd complex stability, thus increasing the free drug amount in solution and allowing the permeation enhancer effect of Cd to be put in evidence.

TEER percent variation and Lucifer Yellow $P_{app}$ values obtained for some representative transport experiments are reported in Table 2. Measurements of the TEER were performed in order to verify the tightness of the junctions between cells [31] and as an aid in interpreting the results of drug permeation studies. No cellular damage occurred during the experiments, since the TEER values never were below 200 $\Omega \times cm^2$, indicative of the integrity of the cell monolayers [45]. CS was able to both significantly ($P < 0.05$) decrease the TEER of Caco-2 monolayers and increase the hydrophilic marker permeability, probably as a consequence of tight junctions opening [11]. These findings seem to indicate that the GB permeation enhancement obtained in the presence of CS could be attributed to the polymer effect on the tight junctions. In contrast, Cds alone were ineffective, at all the concentrations used, in opening epithelial tight junctions, as demonstrated by unchanged ($P > 0.05$) TEER values and lack of enhancer effect of the marker flux. On the other hand, possible extraction of cellular membrane components [39,41,46] or inhibitory effects on drug efflux phenomena mediated by P-glycoprotein [40,42] could be responsible for the enhancing effect of Cd on GB absorption. In particular, GB is, as tacrolimus [42], a substrate of P-glycoprotein [47].

Table 2
TEER at 120 min (% of the initial value) and Yellow Lucifer (Y.L.) $P_{app}$ values across the Caco-2 cell monolayer during the transport experiments at 37 °C of glyburide (GB) alone or in the presence of 0.0625% w/v chitosan (CS) or different βCd or HPβCd concentrations, with or without 0.0625% w/v CS (standard deviations in brackets)

| Sample | TEER (%) | Y.L. '$P_{app}$ $10^{-6}$ (cm/s)[a] |
|---|---|---|
| GB alone | 102 (19) | 1.77 (0.05) |
| GB + CS | 69 (12)[b] | 2.31 (0.27)[b] |
| GB + 7.5 mM βCd | 110 (19) | 1.64 (0.06) |
| GB + 7.5 mM βCd + CS | 73 (13)[b,c] | 2.69 (0.29)[b,c] |
| GB + 25 mM HPβCd | 105 (18) | 1.81 (0.04) |
| GB + 25 mM HPβCd + CS | 77 (14)[b,c] | 2.71 (0.14)[b,c] |

[a] Mean ± SD ($n = 6$).
[b] Significantly different ($P < 0.05$) from GB alone.
[c] Significantly different ($P < 0.05$) from the corresponding sample without CS.

## 4. Conclusion

This study has demonstrated that both βCd and its highly hydrosoluble hydroxypropyl-derivative are clearly more effective in enhancing the GB dissolution behavior with respect to the hydrophilic but poorly water-soluble CS. Moreover, when using the carriers together, a possible competition effect was observed in solubility studies, with a consequent reduction of cyclodextrin solubilizing efficiency towards GB.

On the contrary, the presence of Cds, at all the tested concentrations, did not significantly increase the GB permeability across Caco-2 cells, whereas CS was distinctly more efficacious enabling a 1.6-fold increase of the drug apparent permeability.

Furthermore, conversely from that observed in solubility studies, permeation experiments revealed a significant synergistic effect of Cd and CS in enhancing drug permeability. Considering that in all transport experiments the drug was not in suspension but in solution, a possible explanation of such results could be the marked decrease of the GB–Cd complex stability constant observed in the presence of CS. In fact it has, as a consequence, an increase of the free drug amount available for permeation, and thus, it could render the enhancing effect of Cd on GB permeation evident.

Further studies are in progress in order to verify these hypotheses and clarify the mechanisms responsible for these findings. However, the obtained results suggest that the simultaneous presence of both these carriers, at suitable relative concentrations, could be opportunely exploited to develop suitable oral dosage forms able to simultaneously improve GB solubility and permeability, and, consequently, enhance and make less variable its bioavailability.

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