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Preparation of celecoxib-dimethyl-β-cyclodextrin inclusion complex: characterization and in vitro permeation study

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Abstract

The ability of 2,6-di-O-methyl-β-cyclodextrin (DM-β-Cyd) to include the anti-inflammatory drug celecoxib (CCB) was evaluated. The complex was prepared by kneading and freeze-drying methods and was characterized in the solid state and in aqueous solution. Water solubility and dissolution rate of CCB, in a medium simulating gastric fluid, significantly increased after complexation, with complete dissolution obtained after 30 and 180 min for the freeze-dried and kneaded complexes respectively. Phase solubility studies showed Ap-type diagrams. Stability constants for the 1:1 and 1:2 CCB-DM-β-Cyd complexes and ^{1}H-NMR studies suggested a probable 1:1 inclusion complex and only an external interaction for the second Cyd molecule. Thermodynamic parameters of the binding process showed the existence of van der Waals forces between CCB and DM-β-Cyd. DM-β-Cyd influenced the permeation of CCB through the CaCo-2 cells monolayer. The increase of permeation observed was due to the fast dissolution rate of the included drug and to a destabilizing action exerted by the macrocycle on the biomembrane.

Keywords: Celecoxib; 2,6-di-O-Methyl-β-cyclodextrin; Chemical–physical characterization; Permeation through CaCo-2 cells

1. Introduction

Celecoxib (CCB) is the first member of the Coxib family, a new non steroidal anti-inflammatory drug (NSAID) class, able to selectively inhibit cyclooxygenase-2 (COX-2). This enzyme is responsible for the prostaglandin biosynthesis involved in the inflammatory response [1]. Non specific NSAIDs act by inhibiting both COX-2 and COX-1, which are essential for the regulation of homeostasis in many tissues. The disruption of COX-1 activity is responsible for various side effects, such as gastrolesivity [2] and interference with platelet function [3]. CCB shows high efficacy in the treatment of osteoarthritis and rheumatoid arthritis, comparable to common NSAIDs, such as naproxen and diclofenac [4,5], and as a result of its high specificity for COX-2, no side effects were observed at therapeutic concentrations.

CCB (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide), as a consequence of its chemical structure, shows high apolar characteristics (Fig. 1). The amide group is weakly acidic with a *p*Ka value of about 11. For this reason CCB is practically insoluble at physiological pH. No liquid formulations exist and the only oral dosage form presently available commercially is a capsule. The oral bioavailability obtained compared to an oral suspension was about 75% and the t_{max} was about 3 h [5].

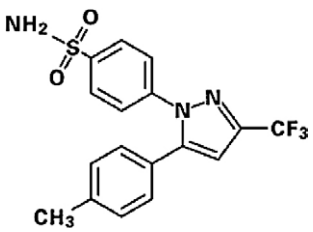

Fig. 1. Chemical structure of CCB.

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By increasing the water solubility of the drug and thus enhancing its dissolution rate, the biopharmaceutical parameters of CCB could be improved. More rapid and quantitative absorption through the gastro-intestinal tract could be observed. To achieve these objectives, cyclodextrins (Cyds), cyclic oligosaccharides, can be used because they possess an external hydrophilic surface and a lipophilic interior. This property of Cyds has been used for 30 years to realize inclusion complexes with various apolar substances to modify the physicochemical characteristics of the included molecule.

Water solubility and dissolution rate of ursodeoxycholic acid and chenodeoxycholic acid were significatively increased by complexation with natural and modified β-Cyds [6]. In the same way, the inclusion of anti-inflammatory drug 4-biphenylylacetic acid into Cyd cavities produces significative enhancement of water solubility and dissolution rate of the drug, improving its oral bioavailability and reducing gastro-intestinal side effects [7]. Natural Cyds have limited water solubility, but a significant increase in water solubility has been obtained by alkylation of the free hydroxyl groups of the Cyds resulting in hydroxyalkyl, methyl, and sulfobutyl derivatives etc. The complexing ability of Cyd derivatives is significantly modified with respect to natural Cyds, for example 2,6-di-O-methyl-β-cyclodextrin (DM-β-Cyd) shows more affinity for some drugs compared to the parent β-Cyd [8] and thus has a higher solubilizing effectiveness. Recently, a CCB inclusion complex with natural β-Cyd has been reported [9]. The authors showed the existence of a 1:1 molar ratio inclusion complex in aqueous solution. Moreover, solid complexes prepared at different combinations of drug and β-Cyd exhibited a higher rate of dissolution and dissolution efficiency than the corresponding physical mixture and pure drug. Molecular modeling studies performed by other authors on CCB-β-Cyd inclusion complex, demonstrated the formation of a stable 1:2 inclusion complex [10].

In this paper we evaluated the ability of DM-β-Cyd to include CCB. The complex was prepared using various techniques and was characterized in the solid state by differential scanning calorimetry (DSC). Circular dichroism (CD) and ^{1}H-NMR spectroscopy were used to confirm the complexation in aqueous solution. Stability constants of the complex (K) were determined by Higuchi and Connors' method [11] in phosphate buffer solution (PBS, pH 7.0) at various temperatures (25, 37 and 45 °C). Thermodynamic parameters of the complexation process were determined on the basis of the vant'Hoff plot. In vitro studies through the CaCo-2 cell monolayer were performed to evaluate the role of the macrocycle on gastro-intestinal absorption of CCB.

2. Results and discussion

Freeze-drying of CCB-DM-β-Cyd water solution produces an amorphous solid sample in which an excess of Cyd is present, in fact, as demonstrated by HPLC analysis a molar ratio of 1:5 was observed. To reduce this ratio, methanol was used as solubilizing agent for CCB, so freeze-dried sample showed a 1:2 CCB-DM-β-Cyd molar ratio.

The CCB-DM-β-Cyd kneaded and freeze-dried solid samples were characterized in the solid state by DSC analysis. As can been seen in Fig. 2, DSC scans of the samples prepared by both methods are comparable; the disappearance of the CCB fusion peak at 160 °C and the appearance of a new peak at 250 °C is evident. The latter could be attributed to the formation of a new solid phase, which melts at higher temperature with respect to the free drug. As concerns the physical mixtures, prepared both in 1:1 and 1:2 molar ratio (curve D and E, Fig. 2), we observed an unusual trend. Generally, the thermograms of physical mixtures are the overlapping of the pure components; in our case we observed a trend similar to that of the complexes. In particular, the 1:2 physical mixture is practically overlapped by the thermograms of the complexes. In the case of the 1:1 physical mixture we observed a reduction of the intensity of the drug fusion peak and a peak of fusion at 250 °C. From these results, it seems that during heating of the physical mixture a complex could be formed. The presence of a CCB fusion peak in the 1:1 physical mixture could evidence a sub-total interaction. This calorimetric trend of the physical mixtures does not allow us to confirm that the kneading and freeze-drying method produces a solid inclusion complex. In fact, only a physical mixture could be obtained, which complexed during heating. CP MAS ^{13}C-NMR studies on CCB-DM-β-Cyd solid samples are in progress to clarify this hypothesis.

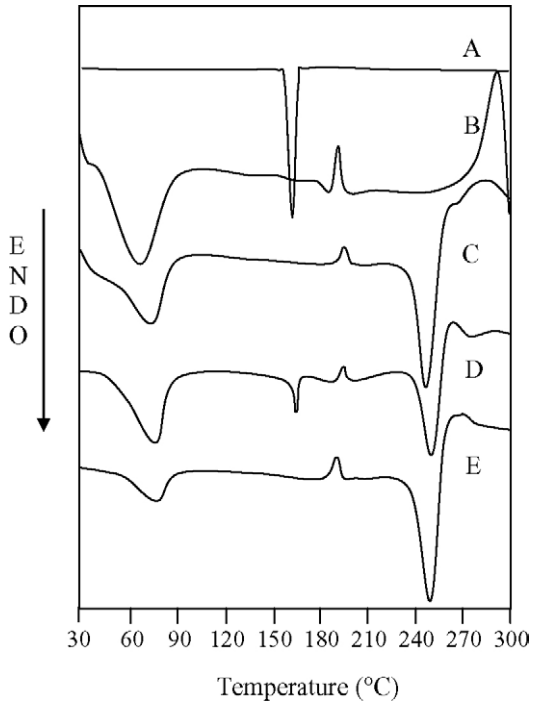


Fig. 2. DSC thermograms of CCB-DM-β-Cyd solid systems. (A) CCB alone; (B) DM-β-Cyd alone; (C) kneaded and freeze-dried CCB-DM-β-Cyd solid sample; (D) CCB-DM-β-Cyd physical mixture (1:1 molar ratio); (E) CCB-DM-β-Cyd physical mixture (1:2 molar ratio).

CD and ^{1}H-NMR spectroscopy were performed to verify the existence in an aqueous solution of the CCB-DM-β-Cyd complex.

CD spectra of CCB alone or in the presence of different concentrations of DM-β-Cyd (CCB/DM-β-Cyd molar ratio 1:10 and 1:100) were shown in Fig. 3. CCB alone does not show CD bands but in the presence of DM-β-Cyd two bands were observed, one negative centered at 240 nm and probably due to $n \rightarrow \pi^*$ transition of the nitrogen group of the pyrazole nucleus, and the other one positive at 280 nm due to $\pi \rightarrow \pi^*$ transition of aromatic groups. The appearance of these CDI bands was due to a perturbation of microenvironment polarity of CCB as a consequence of the inclusion into the DM-β-Cyd cavity. Harata and Uedaira [12] reported that the appearance of CDI bands is due to spatial disposition of the electronic chromophore into the Cyd cavity. A positive band is related to parallel orientation of the dipole moment of the drug with respect to the *Z*-axis of the macrocycle cavity. On this basis, it can be hypothesized that the CCB aromatic group is included in the macrocycle with an axial orientation.

To better evaluate the conformation in solution of the CCB-DM-β-Cyd inclusion complex we performed ^{1}H-NMR studies. The shifts of CCB protons in the presence of DM-β-Cyd in two different molar ratios are shown in Table 1. A significant upfield shift was observed for the H-4 proton, which could be due to the association of this proton with the methoxyl oxygen atoms of DM-β-Cyd, rich in π electrons [13]. Downfield shifts were observed for all aromatic protons, probably as a result of the complexation into the macrocycle cavity, which produces a change in the local polarity [14] or a deshielding effect due to van der Waals forces between the drug and carbohydrate chains [15]. By increasing the molar ratio (CCB/DM-β-Cyd, 1:2), a similar trend to that of the 1:1 molar ratio was observed, but more consistent shifts were

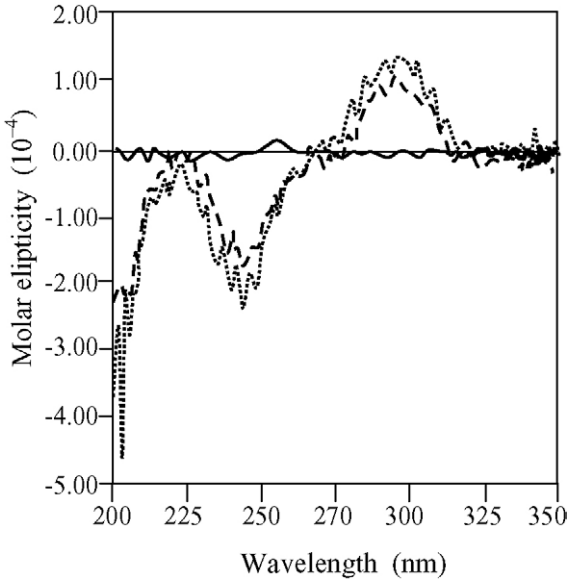
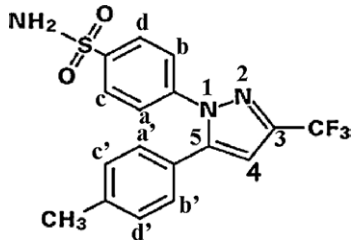


Fig. 3. Circular dichroism spectra of CCB alone (—); CCB in the presence of DM-β-Cyd (1:10 molar ratio) (— —); CCB in the presence of DM-β-Cyd (1:100 molar ratio) (– – – –).

Table 1
^{1}H-chemical shifts of CCB alone and in the presence of DM-β-Cyd in 1:1 and 1:2 molar ratios



Protons	CCB	Δδ* (1:1 molar ratio)	Δδ* (1:2 molar ratio)
H-4	6.989	–0.048	–0.063
a–b	7.497	0.009	0.012
c–d	7.946	0.010	0.09
a′–b′–c′–d′	7.181	0.011	0.025
Methyl	2.331	0.006	0.009

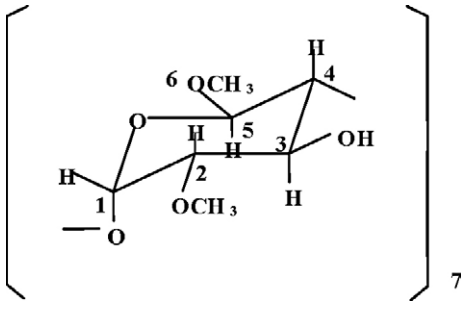
* $\Delta\delta = \delta_{complex} - \delta_{free}$.

observed for the aromatic protons of the toluene group with respect to those in close proximity to the sulfonamide group.

As regards DM-β-Cyd, in the presence of the drug both in 1:1 and 1:2 molar ratio, a significative upfield shift for H-3 and H-5 protons was observed (Table 2). This trend is due to a shielding effect produced by the anisotropic current of CCB aromatic group that was included into the Cyd cavity and suggests that the drug penetrates deeper into the Cyd cavity. The shifts of H-1 and H-4 protons could be due to a change in the macrocycle rigidity due to the complexation. The H-5 proton was not detectable in the 1:2 CCB-DM-β-Cyd complex, because of overlapping Cyd protons.

To verify the spatial disposition of DM-β-Cyd and CCB in the complex we performed NOE experiments (Table 3).

Table 2
^{1}H-chemical shifts of DM-β-Cyd alone and in the presence of CCB in 1:1 and 1:2 CCB- DM-β-Cyd molar ratios



Protons	DM-β-Cyd	Δδ* (1:1 molar ratio)	Δδ* (1:2 molar ratio)
H-1	5.067	0.049	0.057
H-2	–	–	–
H-3	3.871	–0.047	–0.054
H-4	3.614	0.018	0.009
H-5	3.740	–0.046	–
H-6	3.677	0.006	0.014
Methyl-2′	3.576	–0.01	–0.011
Methyl-6′	3.372	0.004	0.009

* $\Delta\delta = \delta_{complex} - \delta_{free}$.

Table 3
NOE effect observed for DM-β-Cyd as a consequence of CCB proton irradiation

Irradiated CCB protons	Intense NOE effect	Weak NOE effect
H-4	6′OCH_3. H-3 and H-5	2′OCH_3
a′–b′–c′–d′	H-3 and 2′OCH_3	H-5 and 6′OCH_3
c–d	None	None
Methyl	6′OCH_3	2′OCH_3. H-5 and H-3

No NOE effect was observed for DM-β-Cyd protons when the CCB protons close to sulfonamide group (c–d) were irradiated, thus confirming that no complexation occurs for this ring. On the contrary, the irradiation of protons of the toluene group (a′–b′–c′–d′) produces a NOE effect for both methoxyl groups and internal protons of the Cyd cavity. In particular, the most effect was observed for H-3 and 2′OCH_3, with respect to H-5 and 6′OCH_3. Moreover, when the CCB methyl group was irradiated, the NOE effect was observed primarily for the 6′OCH_3 of DM-β-Cyd. These results demonstrate the existence in solution of a 1:1 inclusion complex, in which the toluene group of CCB, involved in the complexation was included into DM-β-Cyd from the larger rim, instead of from the other one. The irradiation of the CCB H-4 proton produces a NOE effect on the 6′OCH_3 group of Cyd. We can hypothesize the proximity of another Cyd at the pyrazole nucleus that could be oriented from its narrow rim. Probably, the pyrazole nucleus interacts superficially with this Cyd.

The influence of DM-β-Cyd on water solubility of CCB was measured at different temperatures using the Higuchi and Connors' method [11]. A positive curvature was observed in the isotherms obtained at 25 and 37 °C, implying the formation of higher order soluble complexes at high Cyd concentrations [11] (Fig. 4). At a higher temperature (45 °C) we obtained an Ap-type isotherm until a DM-β-Cyd concentration of 28×10^{-3} M, followed by a plateau due to a decrease of DM-β-Cyd solubility which reduces the solubility of the formed complex.

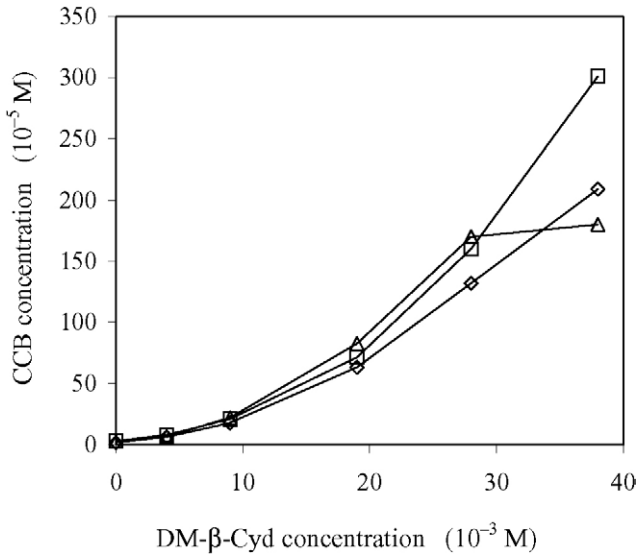


Fig. 4. Solubility phase diagrams of the CCB-DM-β-Cyd system at various temperatures. (◇) 25 °C; (□) 37 °C; (Δ) 45 °C.

Assuming that Ap-type diagram results from the formation of two complexes with 1:1 and 1:2 CCB-DM-β-Cyd molar ratios, stability constants (K) for the two complexes were determined after constructing a plot by using the following Eq. [11]:

$$([S_t] - [S_0])/[L_t] = K_{1:1}[S_0] + K_{1:1}K_{1:2}[S_0][L_t]$$

where $[S_t]$ is the total drug concentration at total Cyd concentration ($[L_t]$), $[S_0]$ is the solubility of CCB in the absence of Cyd. A plot of $([S_t] - [S_0])/[L_t]$ vs. $[L_t]$ results in a linear plot with an intercept of $K_{1:1}[S_0]$ and a slope of $K_{1:1}K_{1:2}[S_0]$ [16]. The K values obtained at different temperatures are shown in Table 4.

It is evident that at 25 °C the 1:1 complex was formed more easily than that at 1:2 molar ratio (K values were 9004 and 141 M^{-1} for the complex in 1:1 and 1:2 molar ratio, respectively). This result was in agreement with ^{1}H-NMR studies that demonstrated the presence in solution of a 1:1 inclusion complex, with a second Cyd molecule probably associated superficially only. Loftsson et al. [17] demonstrated that Ap-type diagrams not necessarily evidenced the presence in solution of complexes at higher order than one in Cyd and the increase of drug solubility could be do to a non-inclusion interaction.

At the increase of the temperature a reduction of $K_{1:1}$ value was observed, showing that 1:1 complexation is an exothermal process. An opposite trend was observed for the 1:2 CCB-DM-β-Cyd interaction, that is an enhancement of K value at the increase of the temperature, evidencing a different interaction of CCB with the second DM-β-Cyd molecule.

Thermodynamic parameters of the binding process between CCB and DM-β-Cyd were determined on the basis of the dependence of the $K_{1:1}$ and $K_{1:2}$ values on temperature. The van't Hoff plots was obtained by plotting log K against the reciprocal of the absolute temperature. The changes in enthalpy (ΔH) and the changes in entropy (ΔS) accompanying the complexation were determined from the slope and intercept of the straight line obtained. Each value obtained is given in Table 5.

The formation of the 1:1 inclusion complex is favored by an enthalpic contribution, rather than an entropic one. High negative ΔH and ΔS were observed. In this way classical

Table 4
Stability constants values ($K_{1:1}$ and $K_{1:2}$) determined for 1:1 and 1:2 CCB-DM-β-Cyd complexes in PBS (pH 7), at different temperatures

Temperature (°C)	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-1})
25	9004.83	141.20
37	343.52	363.95
45	109.60	688.602

Table 5
Thermodynamic parameters of the CCB-DM-β-Cyd binding process

	ΔH (cal mol^{-1})	ΔS (cal K^{-1} mol^{-1})	ΔG (cal mol^{-1})
1:1 molar ratio	–42.51	–124.58	–5.39
1:2 molar ratio	14.94	59.88	–2.93

hydrophobic interactions can be excluded as driving forces for complexation because they are characterized by a positive ΔH and a large positive ΔS [18]. The favorable ΔH and unfavorable ΔS observed for 1:1 CCB-DM-β-Cyd inclusion complex can be due to van der Waals forces between host and guest. Moreover, the negative ΔH can be also explained by a release of enthalpy-rich water molecules from the Cyd cavity [19]. These water molecules cannot form their hydrogen-bonding potentials, thus their displacement from the cavity by suitable guest molecules with less polarity than water could result in a favorable drop in enthalpy. As concerns the entropy changes, they are due to the disordering of the water layers that surround the complex after interaction [20] and because these layers are less ordered or contain a smaller number of water molecules with respect to free reactants, ΔS should be positive. Probably the negative ΔS value can be ascribed to a more conformational rigidity of the drug and the macrocycle due to complexation. On the other hand since methylated Cyds are highly surface-active, other factors, such as micellar dissociation of the host molecules, should be considered for ΔH and ΔS changes.

For what concern the interaction of the 1:1 complex with the second Cyd molecule, we easily observed that this process was entropically favorite. High positive ΔS and positive ΔH were in fact obtained. The positive ΔH can be justified by the presence of an external and/or superficial interaction that no produce the displacement of water molecule from the Cyd cavity. About positive ΔS probably it reflects a less order of the resulting systems. Molecular modeling studies are in progress to verify the conformation of the 1:1 and 1:2 complexes.

Complexation of CCB into the DM-β-Cyd cavity produces a significative increase of the drug water solubility (4.12×10^{-3}, 0.5 and 0.382 mg ml^{-1} at 25 °C for free CCB and CCB as 1:2 freeze-dried and kneaded complex, respectively). The enhanced solubility positively influences the dissolution rate of CCB (Fig. 5). In fact, both freeze-dried and kneaded complexes dissolved more rapidly than the free drug and the physical mixture (1:2 molar ratio). The increase of wettability and the reduction of CCB molecular crystallinity observed after complexation can also be considered in the dissolution process. Quantitative dissolution was reached within 30 min in the case of the freeze-dried complex; this method produces an amorphous CCB-DM-β-Cyd complex, characterized by a very rapid dissolution.

2.1. In vitro permeation study

To evaluate the effect of complexation on oral bioavailability of the included CCB, an in vitro study was performed using a CaCo-2 cell monolayer as intestinal absorption model. The monolayer was mounted on Franz cells and the receptor phase was sampled within 4 h, to assay permeated CCB. Cell vitality was detected at the end of the experiment by transepithelial electrical resistance (TEER) measurement. Permeation studies were performed using the CCB-DM-β-Cyd com-

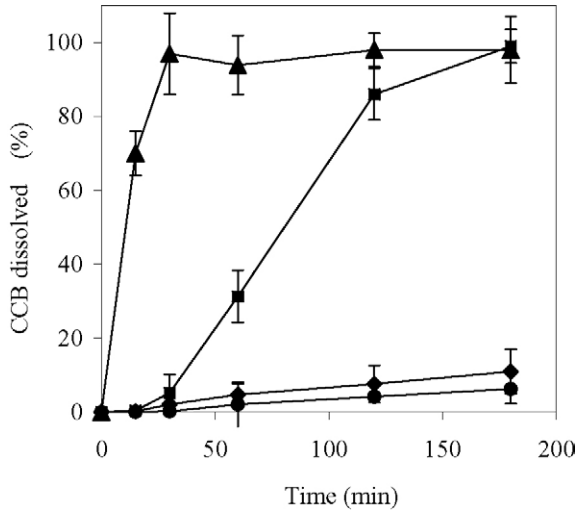


Fig. 5. Dissolution profiles of the CCB-DM-β-Cyd complex. (●) CCB alone; (✦) CCB-DM-β-Cyd physical mixture in 1:2 molar ratio; (■) CCB-DM-β-Cyd kneaded complex; (▲) CCB-DM-β-Cyd freeze-dried complex.

plex prepared in 1:2, 1:5 and 1:10 molar ratios comparatively to a suspension of the freeze-dried drug alone. As observed in Fig. 6, there was a significative increase of total CCB permeated through the membrane when the drug was complexed with DM-β-Cyd. This trend can be due to different factors: (i) rapid dissolution of the complex with respect to CCB alone, which enhances the availability of the drug in solution to the absorption site; (ii) destabilizing action of the macrocycle on the biomembranes. Our studies using liposomes as biomembrane models [21] demonstrated that Cyds are able to increase drug permeability through biomembranes by the extraction and complexation of the lipidic components of cells (phospholipids and cholesterol). This action concerns only free Cyds [22] and it is counterbalanced by its

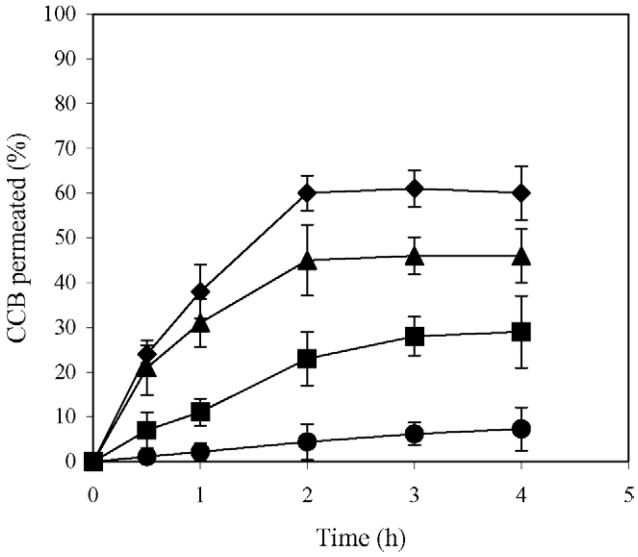


Fig. 6. Permeation profiles of CCB alone or in the presence of the CCB-DM-β-Cyd complex in a different molar ratio. (●) CCB alone; (■) CCB-DM-β-Cyd 1:2 complex as suspension; (▲) CCB-DM-β-Cyd 1:5 complex as solution; (✦) CCB-DM-β-Cyd 1:10 complex as solution.

affinity (measured by stability constant values) for a drug eventually present in the same medium.

In the case of the CCB-DM-β-Cyd complex prepared in a 1:2 molar ratio the sample was applied on the monolayer as a suspension, such as a free drug. In this condition both fast dissolution rate and enhancing action of the Cyd can influence the permeation of the drug. The dissolution probably plays the major role because, due to a high *K* value, the higher amount of Cyd is involved in the complexation and only a small amount of the macrocycle was free to act on the biomembranes. To exclude the influence of dissolution rate of the complex on CCB permeation, we performed the study on samples prepared at a high CCB-DM-β-Cyd molar ratio. In this condition the sample was in solution and the unique factor that can influence the permeation was the destabilizing action exerted by Cyd on CaCo-2 cells. Obviously, the higher concentration of free DM-β-Cyd was related to higher permeation profiles, thus, in the presence of the 1:10 complex 60% of CCB permeates through the monolayer within 2 h After this time a plateau was observed for both 1:5 and 1:10 complexes. Because a saturation in CCB of the acceptor phase can be excluded for the presence of DM-β-Cyd (see Section 4), this plateau could be exploited on the basis of an equilibrium obtained as a result of a probably active mechanism that is involved in the transport of CCB through the biomembrane.

3. Conclusions

The ability of DM-β-Cyd to include CCB was evaluated both in the solid state and in aqueous solution. In the solid state the similar DSC thermograms obtained for the physical mixture and solid samples do not verify the validity of the freeze-drying and kneading methods to produce a solid complex; only a physical mixture could be produced that complexed during heating. Circular dichroism spectroscopy and NMR studies demonstrated a deep inclusion of the toluene group of the drug into the Cyd cavity and a superficial interaction of the pyrazole nucleus with another Cyd. Phase-solubility studies showed Ap-type diagrams at all temperatures considered, that implied the presence in solution of two CCB-DM-β-Cyd complexes in 1:1 and 1:2 molar ratios. The 1:1 complex was formed more easily than that at 1:2 molar ratio, at 25 °C (*K* values were 9004 and 141 M^{-1} for the complex in 1:1 and 1:2 molar ratio, respectively). The binding process of 1:1 CCB-DM-β-Cyd inclusion complex was favored by an enthalpic contribution rather than an entropic one, justified by van der Waals forces between the host and guest molecules and release of enthalpy-rich water molecules from the CyD cavity. The 1:2 CCB-DM-β-Cyd complexation was entropically driven, evidencing a different interaction of CCB with the second Cyd molecule.

The complexation produced a significative increase of water solubility and dissolution rate of CCB; particularly the freeze-dried solid sample, which totally dissolved within 30 min. DM-β-Cyd is able to increase the permeation of CCB across a CaCo-2 cell monolayer mounted in Franz cells, with respect to the free drug. Two mechanisms were invoked in this process: (i) the increase of water solubility of included CCB and (ii) the destabilizing action exerted by Cyd on the biomembrane.

4. Experimental

4.1. Materials

CCB was obtained by repeated extractions with methanol from a marketed formulation (capsule—Solexa®; Pfizer) and the purity was assayed by ^{1}H-NMR and elemental analysis (Anal. Calcd. for $C_{17}H_{14}F_3N_3O_2S$: C, 53.54; H, 3.7; N, 11.01; S, 8.39; found: C, 52.99; H, 3.81; N, 10.84; S, 8.91). DM-β-Cyd was purchased from Cyclolab R&D Laboratory (Budapest, Hungary) and used without further purification.

All other chemicals and solvents were of analytical reagent grade. De-ionized double-distilled water was used throughout the study.

4.2. Preparation of the CCB-DM-β-Cyd complex

A solid complex of CCB with DM-β-Cyd was prepared by the kneading and freeze-drying method. *Kneading*: A mixture of CCB (28.6 mg; 7.5×10^{-2} mmol) and DM-β-Cyd (200 mg; 15×10^{-2} mmol), in a 1:2 mol ratio, was wetted with a methanol/water solution (4:6 v/v) and kneaded thoroughly for 30 min. During kneading, drops of the solvent were added to the solid mixture in order to maintain its consistency as a paste. The obtained sample was dried under reduced pressure at 25 °C, for 1 day. *Freeze-drying*: DM-β-Cyd (500 mg; 37.5×10^{-2} mmol) was solubilized in 30 ml of water or water/methanol solution (50:50 v/v) at room temperature and added to an excess amount of solid CCB. The suspension was stirred at room temperature for 2 days until equilibrium was reached. The suspension was then filtered through a 0.45 μm Millipore filter and the filtrate was freeze-dried (EDWARDS, Modulyo 4 K).

An amount of the solid samples (10 mg) obtained with the freeze-drying method was solubilized in methanol (5 ml) and analyzed by HPLC to determine the drug-Cyd molar ratio.

4.3. Differential scanning calorimetry (DSC)

DSC scans were recorded on a Mettler DSC 12E equipped with a Haake thermocryostate mod. D8-G. A Mettler TA89E and FP89 system software was used for the data acquisition. Indium was used to calibrate the instrument. Each sample was scanned at a speed of 10 °C min^{-1} in the 30–300 °C temperature range.

4.4. Circular dichroism (CD) spectra

CD spectra were performed on a Jasco J-600D recording spectropolarimeter. Free CCB or CCB in the presence of dif-

ferent concentrations of DM-β-Cyd (1:10 or 1:100 molar ratio) were solubilized in a water/methanol solution (70:30, v/v) and stirred before the analysis for 12 h.

4.5. 1H-NMR studies

^{1}H-NMR spectra were recorded, at a probe temperature of 303°K, on a VARIAN Unity Inova Instrument at 200 MHz. For the analysis, CCB (0.4 mg) and DM-β-Cyd in a molar ratio of 1:1 and 1:2 were poured into vials and added to 1 ml of D_2O/CD_3OD solution (50:50, v/v). After stirring for 24 h, 0.7 ml of the obtained solutions were submitted to analysis. Free CCB and DM-β-Cyd were solubilized in the same solvent mixture. No internal standards were added to the samples due to their interaction with the Cyd cavity, the residual sign of CD_3OD at 3.3 ppm was used as reference. To evaluate the probable spatial disposition of the complex, NOE experiments were performed on the 1:2 complex by using a VARIAN Unity Inova Instrument at 500 MHz. The sample was solubilized in D_2O/CD_3OD solution (50:50 v/v).

4.6. Water solubility and dissolution rate determination

To determine water solubility free CCB and of the complexes prepared by different methods, excess amounts of each sample were suspended in 2 ml of water and stirred at room temperature for 2 days. The suspensions were then filtered (0.45 μm Millipore filter) and analyzed by HPLC.

Dissolution rates of free CCB and CCB-DM-β-Cyd kneaded and freeze-dried complexes were carried out according to the U.S.P. 25° paddle method. An amount of 50 mg of free CCB or a corresponding amount in complexes and physical mixture were suspended in 900 ml of pH 1.1 hydrochloride solution and stirred at 100 rpm at 37 ± 0.5 °C. At fixed time intervals, samples were withdrawn and the concentration of CCB in solution was analyzed by HPLC. The experiments were carried out in triplicate.

Free CCB was freeze-dried before using by the same procedure employed to prepare CCB-DM-β-Cyd solid sample.

4.7. Solubility studies

Solubility phase diagrams of CCB-DM-β-Cyd system were obtained by the Higuchi and Connors' method [11]. Excess amounts of CCB were added to PBS (pH 7.0), poured into flasks, containing various concentrations of DM-β-Cyd ($0–38 \times 10^{-3}$ M) and shaken for 2 days at various temperatures (25, 37 and 45 °C). The suspensions were filtered through a 0.45 μm Millipore filter and CCB concentration was determined by HPLC.

Thermodynamic parameters were evaluated using the vant'Hoff plot, on the basis of the dependence of the stability constant (K) on temperature.

4.8. HPLC analyses

HPLC analyses were performed at room temperature using a 1050 Hewlett Packard apparatus on a 5 μm Hypersil ODS cartridge (125 × 4 mm I.D.) (Hewlett Packard) equipped with a 5 μm Hypersil 100 RP-18 guard cartridge (4 × 4 mm I.D.) (Hewlett Packard) and eluted isocratically with acetonitrile/water (55:45, v/v). The flow rate was fixed at 1 ml min^{-1} and the UV detection wavelength used was 252 nm.

4.9. Permeation experiments through CaCo-2 cells

In vitro permeation studies were performed using CaCo-2 epithelial cells (Zooprophylactic Institute of Lombardia and Emilia Romagna) used between passage 28 and 45. They were seeded into Snapwells™ (1 cm^2, 0.4 micrometer pore size: Costar Corporation, Milan, Italy) at a density of 10 cm^2. Cells were maintained in a humidified 5% CO_2 air atmosphere at 37 °C and were cultured in DMEM supplemented with 10% FBS, streptomycin (100 micrograms ml^{-1}), penicillin (100 U ml^{-1}), 1% L-glutamine, and 1% NEAA. The growth media was replaced every day. Confluent cell monolayers were obtained 18–22 days after inoculation. The confluence was demonstrated by means of TEER measured with a voltmeter (Millicell ERS). The measured TEER was between 500 and 600 Ω cm^2.

Permeation experiments were performed by means of Franz type diffusion cells, putting the Snapwells™ containing the CaCo-2 cells monolayer in the donor compartment. An aliquot of 200 μl of each sample, containing 1 mg of freeze-dried CCB or the equivalent amount in the complexes (CCB/DM-β-Cyd molar ratio, 1:2, 1:5 and 1:10) in 1 ml of PBS (pH 7.4), was applied to the monolayer. The acceptor phase was PBS (pH 7.4) containing DM-β-Cyd (0.5% p/v) as solubilizing agent for permeated CCB. Samples of the receiving solution were withdrawn at different times during the experimental period (4 h); the sample volumes were replaced with the same amounts of fresh DM-β-Cyd solution. At the end of the experiments the TEER was remeasured. All samples were analyzed by HPLC to determine the concentration of CCB. The obtained values were corrected for the dilution used during the sampling.

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