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ORIGINAL ARTICLE

Inclusion complexes of 5-flucytosine with β-cyclodextrin and hydroxypropyl-β-cyclodextrin: characterization in aqueous solution and in solid state

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Abstract Complexation between 5-flucytosine (5-FC), a cytosine analogue with in vitro antifungal and antiyeast activity, and β-cyclodextrins (β-cyclodextrin and hydroxypropyl-β-cyclodextrin) was studied in solution and in solid states. Complexation in solution was evaluated using solubility studies, UV–vis and ^{1}H-NMR. In the solid state, differential scanning calorimetry (DSC), scanning electron microscopy (SEM), FT-IR and X-ray diffraction studies were used. UV–vis, FT-IR and ^{1}H-NMR spectroscopy studies showed that the complex formed occurs by complexation of piridinique base analogue into inner cavity. DSC studies showed the existence of a complex of 5-FC with β-CDs. X-ray studies confirmed the DSC results of the complex existence. Solubility studies showed that the complexed drug is forty times more soluble than free 5-FC, indicating the obtained systems as future, promising drug carriers.

Keywords Cyclodextrin · 5-FC · Inclusion complex

Introduction

The treatment of life-threatening, deep seated, systemic fungal infections has become difficult in the last decades, because the range of clinically important fungi has broadened, and the number of immunosuppressed patients and the prevalence of resistance to antifungal agents are both increasing [1]. In this context the interest in developing new antifungal agents or reducing the dosage, with the resistance reducing has continuously increased.

Cyclodextrins (CDs) and their derivatives are well-known host molecules, able to form inclusion complexes rather nonspecifically with a wide variety of guest molecules. Complexation process with native or modified CDs increase guest solubility and stability against the effects of light, heat, and oxidation, reduce volatility and mask unwanted physiologic effects [2]. Because of this the most common application of CDs in the pharmaceutical industry is to enhance drug solubility, dissolution rate, and bioavailability of poorly water soluble drugs. A large variety of drugs encapsulated through noncovalent interactions into unmodified or modified CDs (especially hydroxypropyl-β-cyclodextrin (HP-β-CD)) cavity were described [3, 4].

5-Flucytosine (5-FC), a fluorinated analogue of cytosine, the oldest synthetic antifungal agent, received a special attention in the last years, because of increased usage in combination with a number of antifungal agents for lethal invasive mycosis treatment and alone as a possible new therapeutic for colorectal carcinoma [1]. 5-FC usage is limited by its major side effects including hepatotoxicity, causing severe liver necrosis [5], and bone-marrow depression inducing life-threatening leucocytopenia, thrombocytopenia and pancytopenia [6, 7].

Complexation of 5-FC with cyclodextrin offers the possibility to improve the aqueous solubility of 5-FC, without modifying the drug original structure, increasing 5-FC bioavailability, and reducing its toxicity. We synthesized β-cyclodextrin-5-FC (β-CD-5-FC) and hydroxypropyl-β-cyclodextrin-5-FC (HP-β-CD-5-FC) inclusion complexes, in order to make the bioactive compound more available, with the consequent reduction of the dosage, the treatment period, and the gravity of all possible side effects.

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Materials and methods

Materials

5-FC (Fluka), β-CD (Aldrich) and HP-β-CD (Cyclolab) were used as received. Double distilled water was used throughout the study.

Methods

Solubility studies

Solubility studies were carried out according to the Higuchi and Connors method [8]. β-CD or HP-β-CD solutions of different concentrations ranging between 0.5 and 40×10^{-1} M, were added to a supersaturated solution of 5-FC and shaken at room temperature (22 ± 1 °C) for 24 h. After reaching equilibrium, the solutions were filtered. The absorbance of solutions containing different mole fraction of the drug and β-CD or HP-β-CD was measured by UV–vis absorption at 192 nm and the concentration of 5-FC in the each solution was determined with reference to a suitably constructed standard curve.

The apparent stability constant was calculated from the phase solubility diagram slope using the Eq. 1 [9].

$$K1{:}1 = \frac{\text{Slope}}{S_0 \cdot (1 - \text{Slope})} \quad (1)$$

where S_0 is the solubility of 5-FC in the absence of β-CD or HP-β-CD. *Slope* the slope of the experimental phase solubility diagram for β-CD-5-FC and HP-β-CD-5-FC.

Preparation of the solid complex

The inclusion complexes (C_s) were prepared by freeze drying method. Aqueous solutions containing 5-FC and β-CD or 5-FC and HP-β-CD, in a 1:1 molar ratio were obtained by dissolving 7.74×10^{-4} mol 5-FC and 7.74×10^{-4} mol CDs in 25 ml distilled water and continuous stirring for 48 h at room temperature. These mixtures were frozen by immersion in liquid nitrogen and freeze-dried in a Martin Christ, ALPHA 1-2LD Freeze-Drier.

Preparation of the physical mixture (PM) was performed by mixing the powders in a 1:1 molar ratio in a ceramic mortar.

Complex characterization

In solution

1. UV–vis measurements were performed on a Analytik Specord 200 Jena Spectrophotometer.

2. Nuclear magnetic resonance (^{1}*H-NMR*), were performed on a DRX 400 Advance Bruker device, at concentrations equal to 1 mg/mL each sample in DMSO.

In the solid state

1. Differential Scanning Calorimetry (DSC) DSC data were obtained using a Perkin Elmer-Diamond device. Each sample (2–6 mg) was exactly weighed in an aluminum pan and was heated at a rate of 10 °C/min between 30 and 250 °C, under nitrogen gas flow.

2. X-ray diffraction (XRD) XRD patterns were obtained on a Bruker AXS D8 advance X-ray diffractometer, on samples weighted 40–60 mg. Using a higher quantity of pure cyclodextrin, than for pure drug or inclusion complex is the reason for obtaining the high intensity diffraction for the pure host.

3. Scanning electron microscopy (SEM) SEM micrographs were obtained on a Tesla Scanning Electron Microscope. The samples (15–20 mg) were weighed before preparation.

4. Fourier transform infrared spectroscopy (FT-IR) FT-IR spectral studies were carried on FT-IR Bruker Vertex 70, instrument using KBr disc method. Scanning was done from 4000 to 310 cm^{-1}.

Results and discussion

In solution

Phase solubility studies

As already cited in the literature the solubility of 5-FC in water is very low, 0.081 M at 25 °C [3], physical property that can be improved by complexation with cyclodextrins. In Fig. 1 it is shown that the solubility curves obtained for 5-FC in the presence of β-CD and HP-β-CD in distilled water. As it can be seen, 5-FC solubility in cyclodextrin aqueous solutions water presents a linear growth, the resulting linear curve can be classified, as an A_L type (linear positive isotherm), as described in literature [8, 9]. The increase in dissolution rate is significant, more than 40 times for both analyzed systems, which can be explained due to the formation of the complex. The maximum concentration of 5-FC in 4 M cyclodextrin water solution is 34.7 for β-CD and 38.7 for HP-β-CD.

The slopes obtained for the resulting linear curves are 0.85 for β-CD-5-FC and 0.96 for HP-β-CD-5-FC, the differences between slope values explaining the differences between K1:1 values. The apparent solubility constant, K1:1, of each complex was calculated from the correspondent curve of Fig. 1 according to Eq. 1, and it was

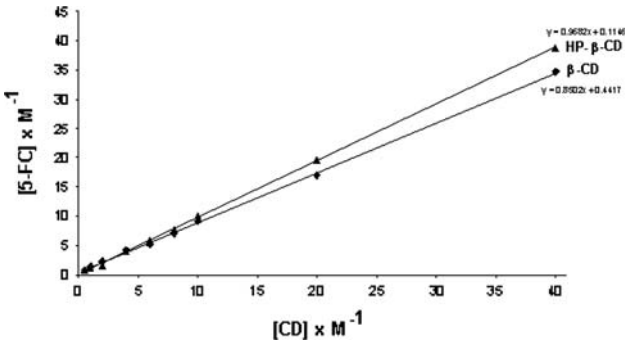

Fig. 1 Higuchi phase solubility diagram of 5-FC in presence of CDs

found to be 70 M^{-1} for β-CD-5-FC and 297 M^{-1} for HP-β-CD-5-FC, values similar to those quoted in the literature for drug cyclodextrin inclusion complexes [10].

UV–vis spectroscopy

UV–vis absorption spectra for the pure drug and the inclusion complexes have been analyzed in distilled water. As it can be seen from Fig. 2, UV–vis spectra of both inclusion complexes show the disappearance of the absorption band at 270–275 nm in water-solution of pure drug due to inclusion complex formation. The similarity of the changes indicates that the inclusion mechanism is similar for the two analyzed cyclodextrins.

^{1}H-NMR spectroscopy

^{1}H-NMR studies reveal useful information on the nature of the interactions between drug and cyclodextrin in inclusion complexes. The comparison of the spectra for the individual components and for the β-CD-5-FC inclusion complex indicates clear differences (Fig. 3, Table 1).

A remarkable downfield shift of H_f2 and H_f1 protons of 5-FC in complex shows that the drug penetrates the β-CD cavity and it is stabilized by forming hydrogen bonds between the protons of NH_2 group with cyclodextrin hydroxyl groups, which are more affected than NH situated on the opposite part of the ring. The order of the shifts is found to be for 5-FC: H_f2 (dδ (ppm) = 0.419) > H_f1 (+0.266) > H_f3 (−0.037). On the other hand, it can be observed major modifications of H_3, H_6, H_2 CD's protons [11, 12] (Table 1). These remarks demonstrate that the inclusion complex is formed, especially by the shifting of H_3 protons situated inside the cyclodextrin cavity (dδ = −0.027).

The same behavior was characteristic for the 5-FC-HP-β-CD inclusion complex, whose spectrum presents clear differences compared to pure 5-FC and HP-β-CD (Fig. 4). In our work only the H_f1 and H_f2 protons of drug can be discussed because in the 3–4 ppm region too many peaks

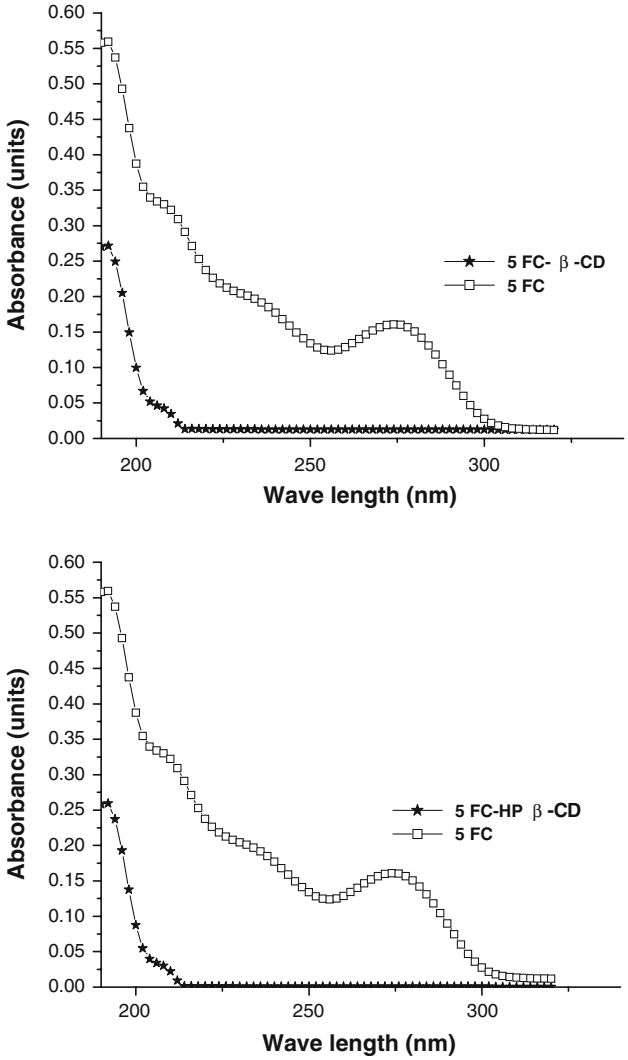

Fig. 2 UV–vis spectra of 5-FC and corresponding inclusion complexes with CDs, in water solution

are overlapped. A downfield shift of the H_f2 protons dδ (ppm) = 0.257 is observed.

These data indicate that β-CD and HP-β-CD are not only suitable for nucleobases separation by forming inclusion complexes [13], cytosine presenting the lowest capacity of forming inclusion complexes [14], but they are also suitable for including 5-FC, with high efficiency, due to Flour presence in drug structure (Table 2).

In solid state

Differential scanning calorimetry

DSC reveals some information on solid-state interactions between drug and cyclodextrins. The DSC thermograms of pure components, physical mixture, and of the 5-FC β-CD inclusion complex are presented in Fig. 5. A typical DSC

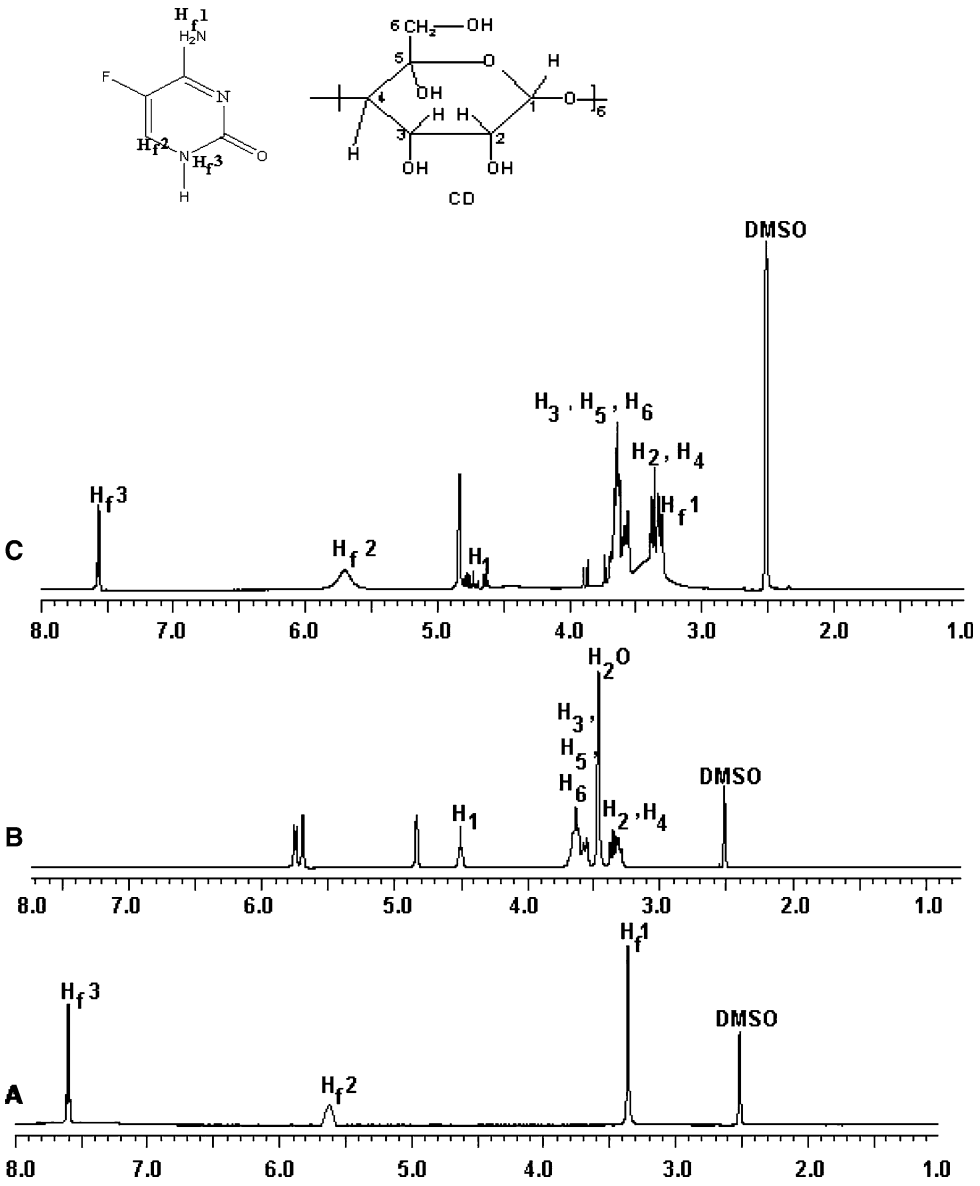

Fig. 3 ^{1}H-NMR spectra for 5-FC (**a**), β-CD (**b**) and β-CD-5-FC inclusion complex (**c**)

Table 1 Chemical shift data (δ in ppm) of H–C protons in 5-FC and β-CD in free state and in the complex state

Code	δ_{free}	$\delta_{complex}$	dδ (ppm)
5-FC			
H_f1	3.359	3.625	+0.266
H_f2	5.278	5.697	+0.419
H_f3	7.607	7.57	−0.037
β-CD			
H1	4.835–4.826/4.8305	4.829	−0.006 to 0.003/0.0015
H2	3.317–3.290/3.3035	3.291–3.300	−0.026 to 0.01/0.265
H3	3.652–3.608	3.356–3.332	−0.296 to −0.276
H4	3.374–3.351	3.323–3.315	−0.051 to −0.036
H5	3.574–3.549/3.5615	3.555	−0.019 to −0.006/−0.0065
H6	3.652–3.608	3.696	0.044 to 0.088

$d\delta = \delta_{complex} - \delta_{free}$

curve for a crystalline anhydrous substance, with a sharp fusion endotherm (T peak = 320 °C), was obtained for 5-FC. The same peak can be observed by analyzing physical mixture thermal behavior. For the complexed drug the characteristic thermal peak appears at 300–305 °C, and it is strongly reduced in intensity and somewhat broadened in complex modification due to inclusion complexation process.

The DSC thermograms of pure drug, of physical mixture, and of 5-FC-HP-β-CD inclusion complexes are presented in Fig. 6. HP-β-CD is an amorphous material, and does not exhibit a melting point, as would be observed for crystalline materials. In physical mixture the characteristic peak for free drug and CD can be easily observed. For the inclusion complex the characteristic thermal peak of 5-FC appears around 270 °C, and it is strongly reduced

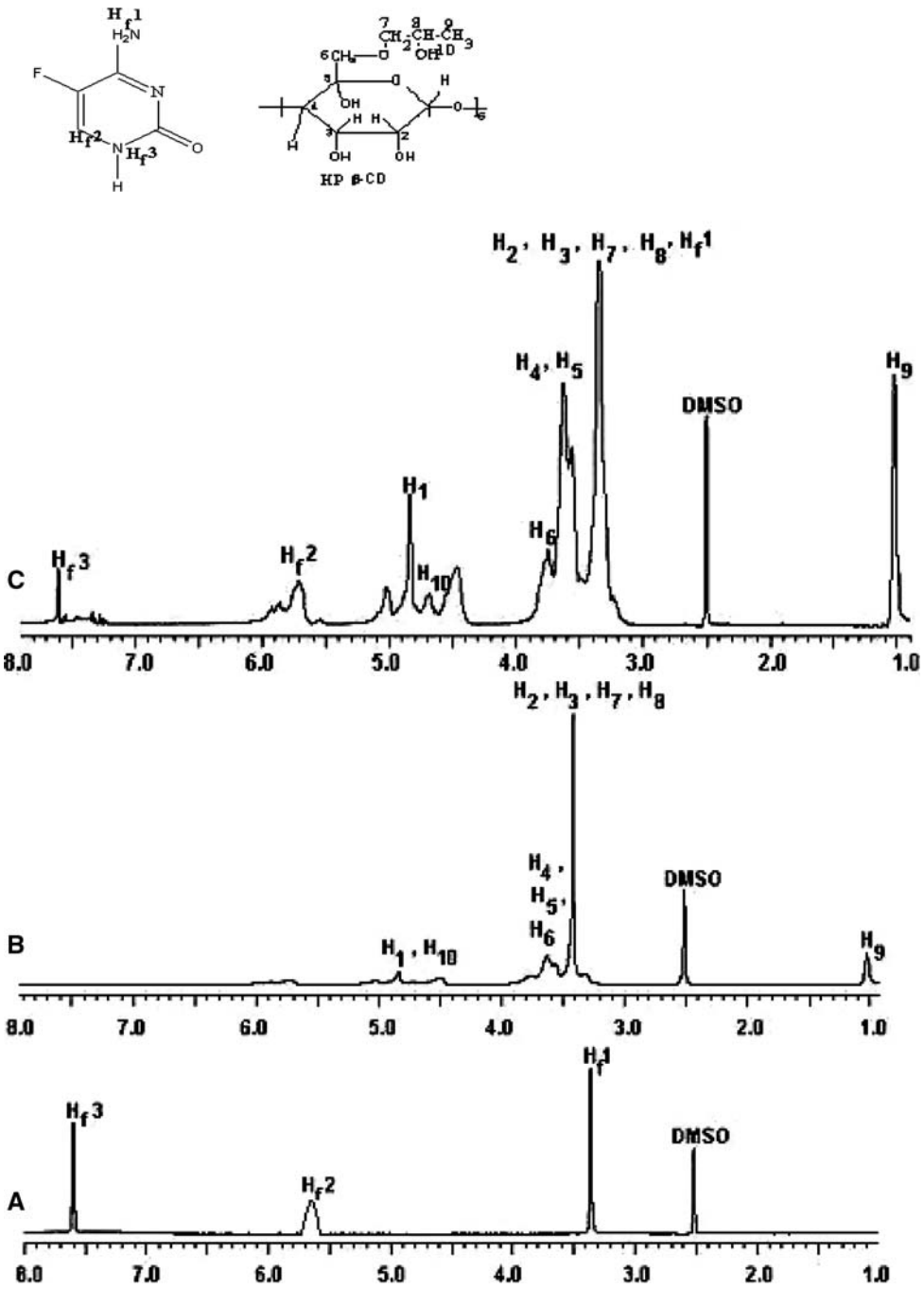

Fig. 4 ^{1}H-NMR spectra for 5-FC (**a**), HP-β-CD (**b**) and HP-β-CD-5-FC inclusion complex (**c**)

in intensity and somewhat broadened in the HP-β-CD-5-FC inclusion complexes due to the inclusion complexation process.

Powder x-ray diffraction

X-ray powder diffraction patterns (XRPD) of pure 5-FC and β-CD, and corresponding solid inclusion complexes with CDs, are shown in Fig.7. The XRPD of β-CD reveals several diffraction peaks, which are indicative of its crystalline character. By contrast, in the complex we could observe a diffraction pattern completely diffuse, which reveals its amorphousness. This behavior can be attributed to an interaction showing the presence of a new solid phase, where a possible formation of an inclusion compound is contemplated [15, 16]. In the X-ray diffractograms of pure components, sharp diffraction peaks are present, indicating their crystalline state. By contrast the X-ray diffraction patterns of 5-FC-β-CD systems were characterized only by large diffraction peaks, in which it is no longer possible to

Table 2 Chemical shift data (δ in ppm) of H–C protons in 5-FC and HP-β-CD in free state and in the complex state

Code	δ_{free}	$\delta_{complex}$	dδ (ppm)
5-FC			
H_f1	3.359	3.348	−0.011
H_f2	5.278	5.024	−0.254
H_f3	7.607	7.608	0.001
HP-β-CD			
H1	4.736–4.766	4.654	−0.082 to −0.112
H2	3.563	3.55	+0.013
H3	3.386	3.29–3.34	−0.096 to 0.046
H4	3.58	3.57–3.628	−0.001 to +0.048
H5	3.629	3.57–3.628	−0.059 to 0.001
H6	3.796	3.751	−0.045
H7	3.37–3.563	3.29–3.34	−0.08 to 0.223
H8	3.37–3.563	3.29–3.34	−0.08 to −0.223
H9	1.012–1.027	1.010	−0.002 to −0.017
H10	4.835	4.84	+0.005

$d\delta = \delta_{complex} - \delta_{free}$

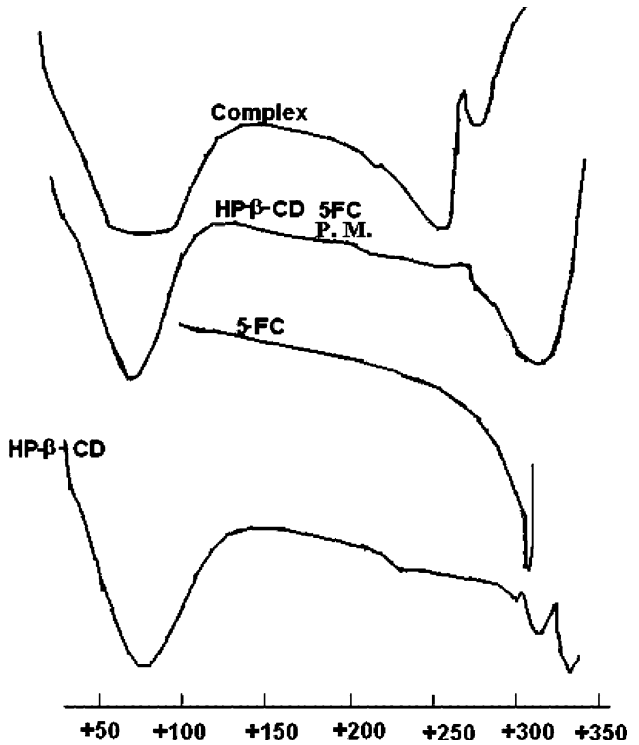

Fig. 5 DSC thermograms of β-CD, 5-FC, physical mixture (PM), and inclusion complex (complex)

distinguish the characteristic crystallinity peaks of pure drug or β-CD. These results indicate that 5-FC is no longer present as a crystalline material, and its CD solid complexes exist in the amorphous state.

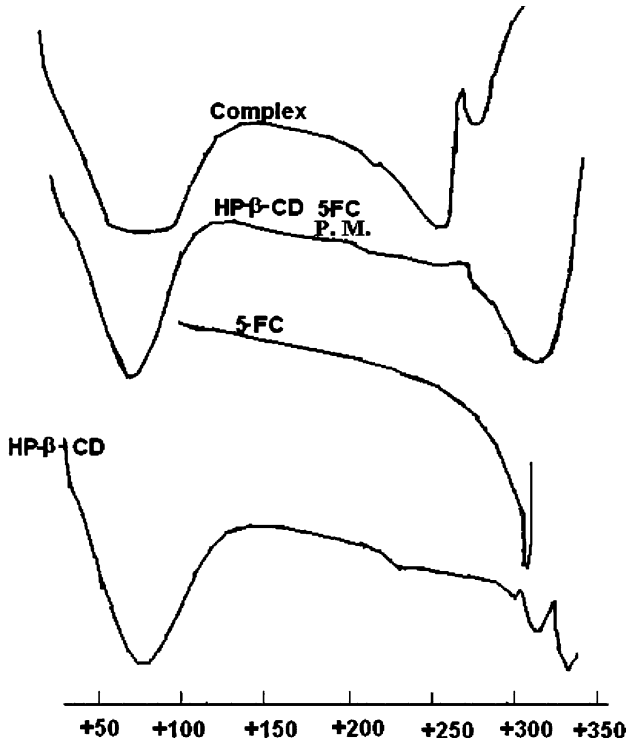

Fig. 6 DSC thermograms of HP-β-CD, 5-FC, physical mixture (PM) and inclusion complex (complex)

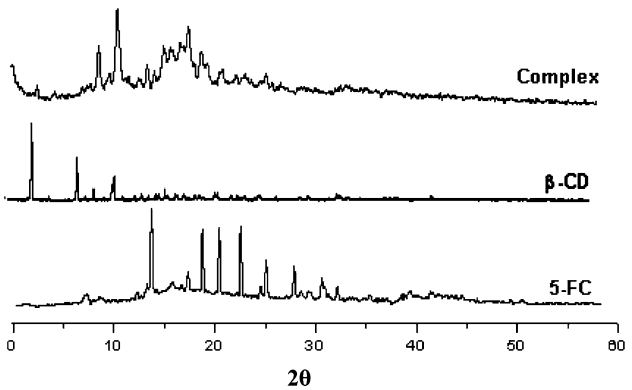

Fig. 7 X-ray diffraction patterns of: 5-FC, β-CD and β-CD-5-FC inclusion complex (complex)

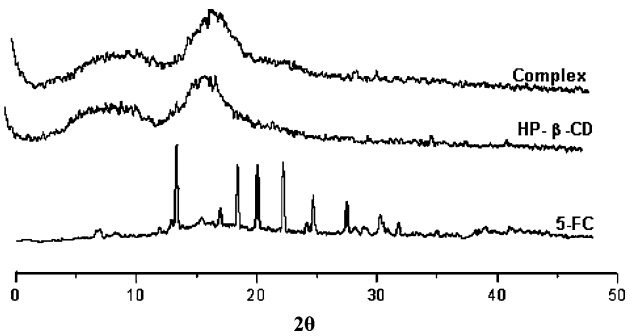

Fig. 8 X-ray diffraction patterns of: 5-FC, HP-β-CD and HP-β-CD-5-FC inclusion complex (complex)

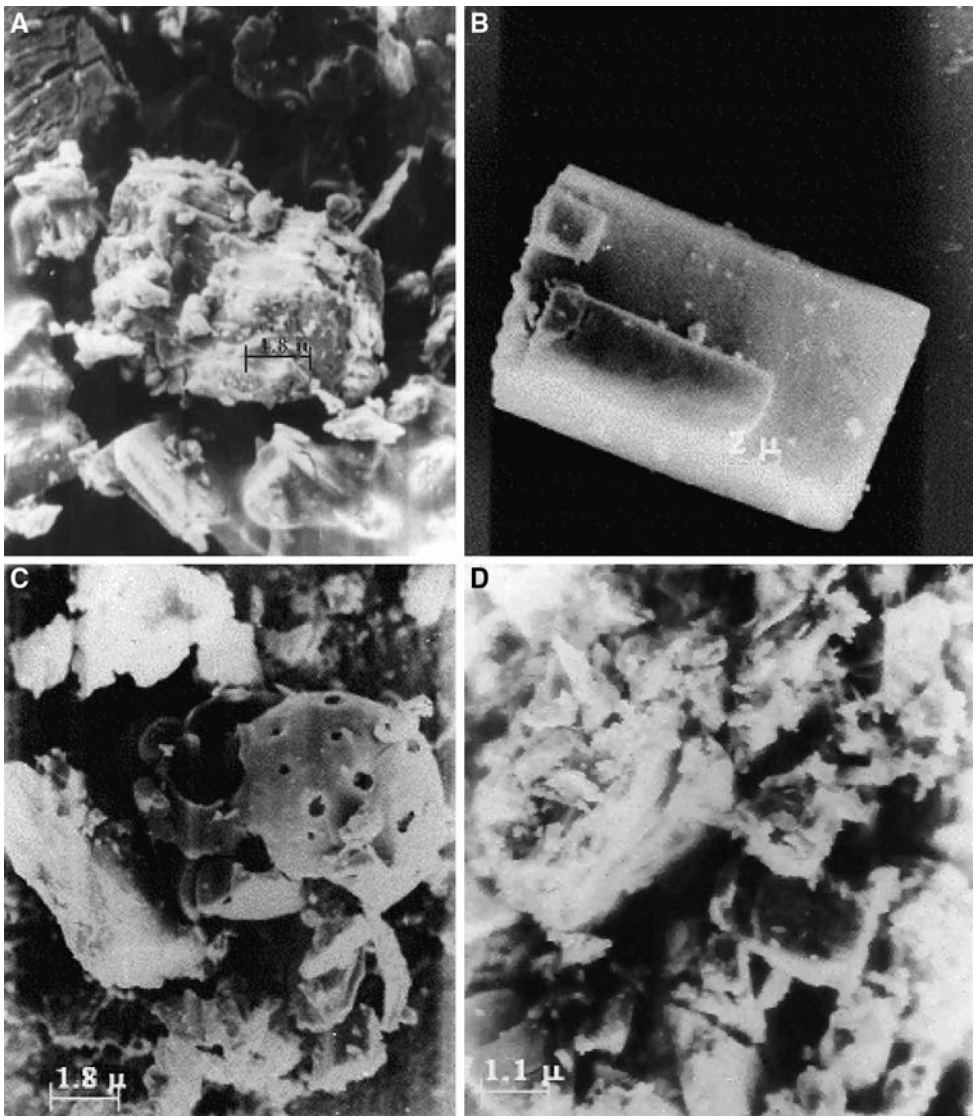

Fig. 9 SEM images of: β-CD (**a**); 5-FC (**b**); physical mixture of β-CD and 5-FC (**c**); inclusion complex (**d**)

The formation of an amorphous state proves that the drug was dispersed in a molecular state with CD [11].

The same behavior can be observed by analyzing HP-β-CD-5-FC inclusion complexes, the amorphization of the drug indicating the complexation efficiency (Fig. 8).

Scanning electron microscopy (SEM)

SEM gives useful information of the complex microstructure, indicating a new crystalline state.

The particle morphology of 5-FC, β-CD, its physical mixture, and solid complex can be seen in SEM photographs presented in Fig. 9. 5-FC appeared as cylindrical crystals, tending to form aggregates. β-CD consisted of cylindrical and irregularly shaped crystals. The physical mixtures showed particles characteristic for β-CD embedded with 5-FC particles. In contrast, the β-CD-5-FC freeze dried complex micrograph shows only one phase, indicating the existence of only one single component, the inclusion complex.

The particular morphology of 5-FC, HP-β-CD, and its physical mixture, and solid complex, can be seen in Fig. 10. Similar to the β-CD-5-FC system, the inclusion complex showed a different crystalline state, contrasting with the physical mixture, in which micrograph the two components are separated. This indicates an apparent interaction in the solid state.

FT-IR spectroscopy

All FT-IR spectra of complexes (Figs. 11 and 12) show changes from parent spectra (i.e. pure drug and CDs). The 5-FC FT-IR spectrum shows the presence of the characteristic peaks: 3375 and 3139 cm^{-1} (NH_2 and NH); 2621 cm^{-1} (due to NH in presence of F in ortho position of pyrimidinique ring); 1671 and 1645 cm^{-1} (C=O, C=N).

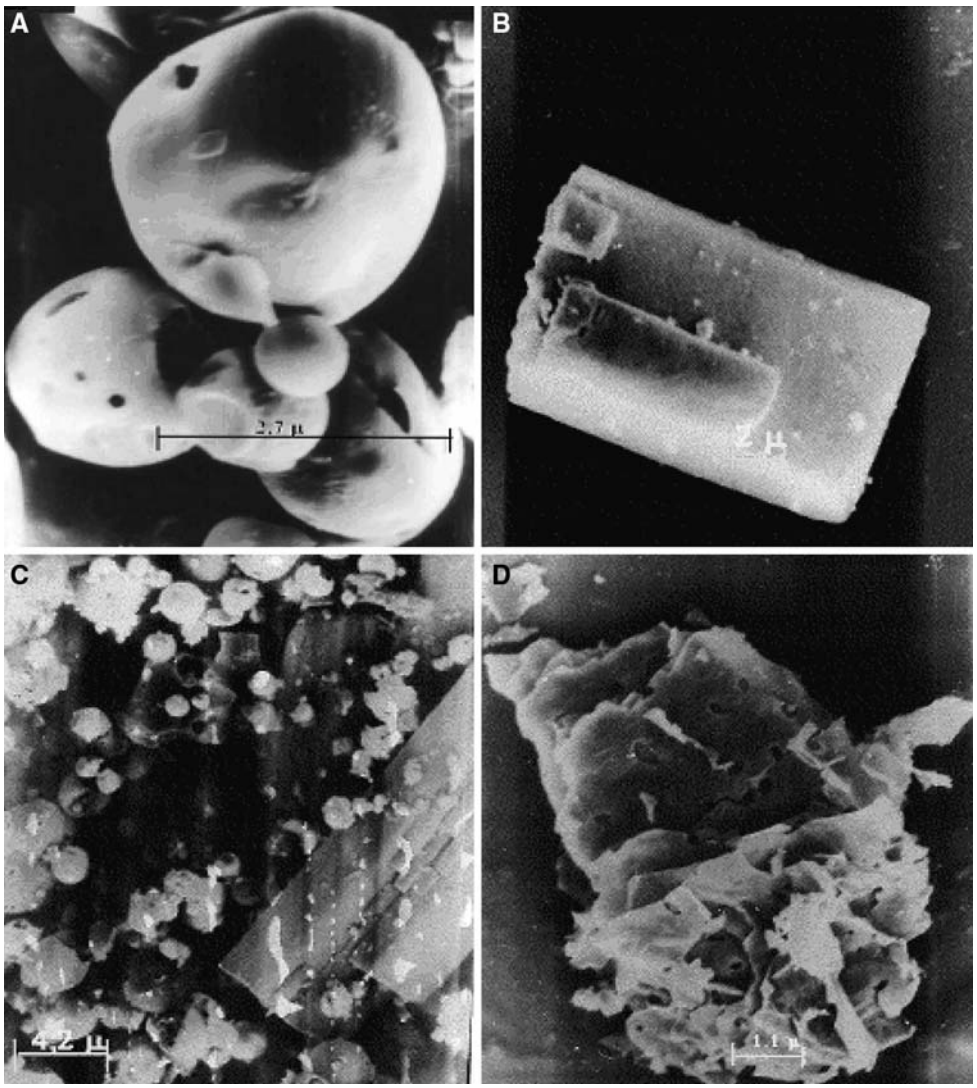

Fig. 10 SEM images of: HP-β-CD (**a**); 5-FC (**b**); physical mixture of HP-β-CD and 5-FC (**c**); inclusion complex (**d**)

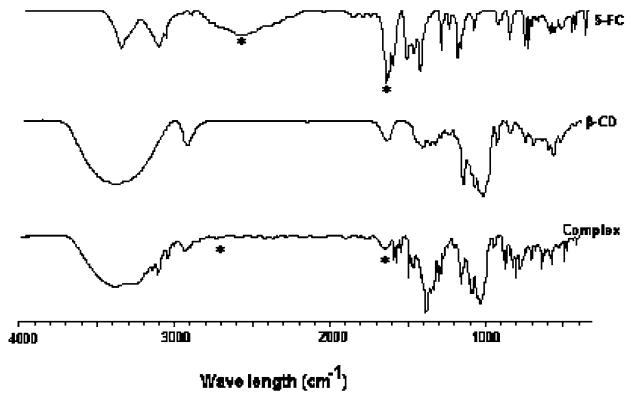

Fig. 11 FT-IR spectra of 5-FC, β-CD, and their inclusion complex (complex)

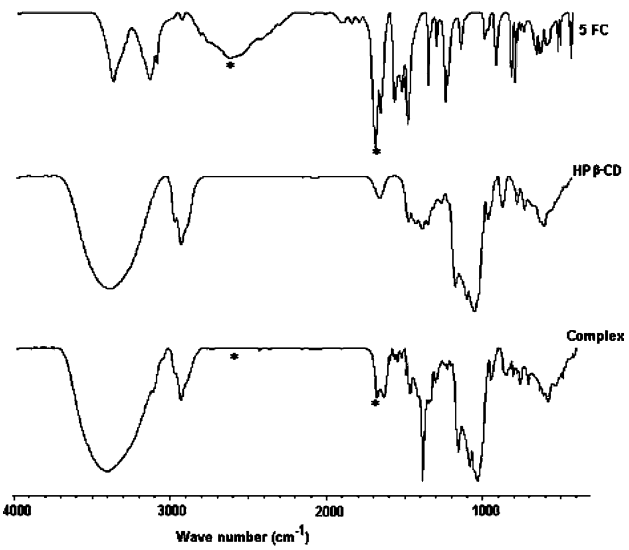

Fig. 12 FT-IR spectra for 5-FC, HP-β-CD, and their inclusion complex (complex)

The FT-IR spectra of β-CD and HP-β-CD show absorption bands at: 3408 and 3351 cm^{-1} (O–H stretching vibration); 2926 cm^{-1} (O–H stretching); 1645 and 1642 cm^{-1} (OH bending); 1356 and 1379 cm^{-1} (OH deformation); 1157 and 1090 cm^{-1} (C–O–C stretching and OH banding); 1028–1006 cm^{-1} and 1056–1028 cm^{-1} (C–O–C stretching). In the FT-IR spectra of 5-FC-β-CDs inclusion complexes the specific peak of drug at 2621 cm^{-1} and 1671 cm^{-1} can not be detected, indicating a strong interaction between the two components. The other peaks of 5-FC are superposed over CDs' peaks.

This indicates that the piridinique base analogue with the F and NH group and the structure is stabilized by hydrogen bonding between the O from 5-FC structure and H from CD hydroxyl groups.

Conclusion

Inclusion complexes of flucytosine and β-CD and HP-β-CD were prepared by freeze-drying method in a molar ratio 1:1. The formation of the inclusion complexes was proved by DSC, SEM, FT-IR and X-ray diffraction methods. From UV–vis spectra the disappearance of the drug characteristic absorption band at 275 nm in inclusion complexes was observed. FT-IR spectra shows that the drug penetrates the CD cavities with the F group (near NH) and the inclusion complex is stabilized through hydrogen bonding implicating NH_2 and O group from 5-FC structure. Complexation by inclusion process increases 5-FC solubility and dissolution rate, almost 43 times in β-CD and 48 times in HP-β-CD presence was observed. Dissolution increasing is due to the low crystallinity of the complex.

Taking into account these results, we can conclude that the interaction of 5-FC with β-CD and HP-β-CD, can lead to important modifications of the physicochemical properties (solubility, stability, bioavailability) of the guest molecule. Therefore, studies are being performed to evaluate the potential biopharmaceutical effects of β-CD-5-FC and HP-β-CD-5-FC products as new drug delivery systems.

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