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# Studies on trimethoprim:hydroxypropyl-β-cyclodextrin: aggregate and complex formation

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ABSTRACT

The present study is focused on the characterization of the interaction between trimethoprim, a dihydropteroate synthesase inhibitor, and hydroxypropyl-β-cyclodextrin (HP-β-CD) in aqueous solution and solid state. The freeze-drying method was used to prepare solid complexes, while simple blending was employed to obtain physical mixtures. The phase solubility was AN type, and demonstrated that trimethoprim solubility was significantly increased upon complexation with HP-β-CD. Conductivity experiments showed the presence of aggregates that explains the type profile for the solubility isotherm. The critical concentration for the aggregate formation was determined to be 69.3 mg/ml for pure HP-β-CD and 117.7 mg/ml in the presence of trimethoprim. Nuclear magnetic resonance spectroscopy provided evidence of trimethoprim:HP-β-CD molecular interaction in solution. Moreover, the complex was characterized in solid stated using Fourier-transform infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM). The use of differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) showed that the thermal stability of the drug is enhanced in the presence of HP-β-CD.



## 1. Introduction

Trimethoprim (Fig. 1) is a synthetic, broad-spectrum antimicrobial agent which acts as an inhibitor of bacterial dihydrofolate reductase. This drug is mainly used in combination with sulfonamides for prophylaxis and treatment of urinary tract and certain types of pneumonia. Trimethoprim is characterized by a very low aqueous solubility[1,2] fact that complicates the preparation of formulations such as those for the intravenous administration.

A strategy widely used to increase the solubility, stability, and bioavailability of drugs is the complex formation with cyclodextrins (CDs).[3–5] CDs are macrocyclic oligosaccharides with six, seven or eight D-glucose units called α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin, respectively. Hydroxypropyl-β-cyclodextrin (HP-β-CD) (Fig. 2) is a hydroxyalkylated β-CD derivative that combines relatively high water solubility with low toxicity and satisfactory inclusion ability.[6,7] The cavities of CDs are relatively hydrophobic, while the external faces are hydrophilic. The most important structural feature of CDs is its capacity to form stable inclusion complexes with properly sized drug molecules,[8,9] prevailing this fact in dilute aqueous solutions.[10] Moreover, it is known that CD molecules have the tendency to self-assemble to form aggregates which can also participate in the solubilization of poorly soluble

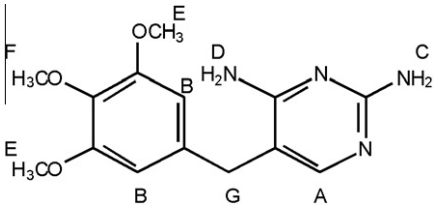


**Figure 1.** Chemical structure of trimethoprim and signal notation.

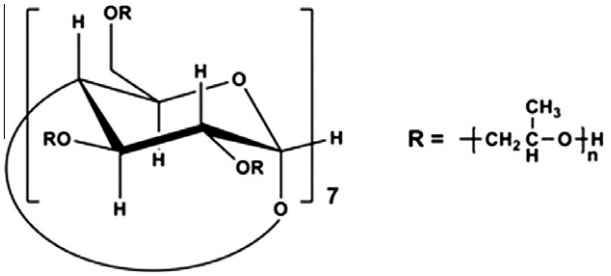


**Figure 2.** Chemical structure of HP-β-CD and signal notation.

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drugs. It was observed that the aggregate formation depends on the concentration. Furthermore, the formation of inclusion complexes with lipophilic guests can convert a CD molecule from a simple oligosaccharide into a molecule capable of forming micellar-type aggregates. In addition, poorly soluble guests can participate in the formation of guest-CD co-aggregates where the molecules are kept together through non-inclusion complexes.[10,11]

Studies involving the complexation of trimethoprim with natural CDs (α-, β- and γ-) and methylated β-CD have been reported in the literature,[12–14] as well as the formation of a 1:1 complex in presence of HP-β-CD at various pH values, which could improve the aqueous solubility of the drug in trimethoprim/sulfamethoxazole parenteral solutions but could not prevent its precipitation.[15] In addition, the chemical stability under oxidation stress of trimethoprim in co-trimoxazole (a 5:1 combination of sulfamethoxazole with trimethoprim) aqueous buffer solutions has been increased using HP-β-CD as a molecular inclusion excipient.[16] Moreover, we have previously developed a method for the simultaneous quantification of trimethoprim and sulfamethoxazole in mixtures using HP-β-CD solutions.[17] Nevertheless, none of the above mentioned works have characterized the inclusion complex of trimethoprim with HP-β-CD and investigated the possibility of aggregate formation, although there were observed results that may be indicative of non-linear complexation.[16]

Considering these previous investigations, the present study aimed to investigate the interactions between trimethoprim and HP-β-CD. The characterization has been performed using solubility analysis, nuclear magnetic resonance ($^1$H NMR), infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), differential scanning calorimetry (DSC), and thermogravimetric analysis (TGA). Also, the formation of aggregates was investigated by conductivity measurements.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All the experiments were performed with analytical grade chemicals and solvents. HP-β-CD, degree of substitution 0.63, was kindly supplied by Ferromet agent of Roquette (France). NMR spectra were taken in deuterium oxide ($D_2O$, deuterium content 99.9%) from Aldrich® USA. A Millipore Milli Q Water Purification System generated the water used in these studies.

### 2.2. Phase solubility studies

The solubility measurements were performed according to the method of Higuchi and Connors.[18] An excess of trimethoprim was suspended in aqueous solutions containing different concentrations of HP-β-CD, ranging from 3.6 to 142.9 mM. Trimethoprim in the absence of HP-β-CD was used to determine the intrinsic solubility. The suspensions were sonicated for 15 min (ULTRASONIC LC 30 H Elma) and then placed for 72 h in a 25.0 ± 0.1 °C constant temperature water bath (Haake DC10 thermostat). These suspensions were sonicated at several time intervals. After the equilibrium was reached, the excess trimethoprim was removed by filtration through a 0.45 μm membrane filter (Millipore, USA). The clear solutions were suitably diluted and analyzed by UV–vis spectrophotometry (SHIMADZU UV-160A spectrophotometer). The absorbance was measured at $\lambda$ = 283 nm.

### 2.3. Conductivity measurements

The conductance measurements were taken in HP-β-CD solutions in absence (with HP-β-CD concentration in the range of 1.33–208.80 mg/ml) and presence of trimethoprim (was used a constant trimethoprim concentration throughout the experiment, and HP-β-CD with a concentration range of 1.65–185.56 mg/ml). All solutions were freshly prepared before each experiment. The critical concentration for the aggregate formation was determined by measuring the specific conductivity change as a function of concentration, using a Malvern Zetasizer 3000 (Malvern Instruments Inc., London, UK). The measurements were performed at 25 °C. The values are the mean of 20 conductance measurements.

### 2.4. Nuclear magnetic resonance (NMR) studies

All experiments were performed on a Bruker® Avance II High Resolution Spectrometer, equipped with a Broad Band Inverse probe (BBI) and a Variable Temperature Unit (VTU). All experiments were carried out at 298 K, using 5 mm sample tubes. The NMR data were processed with the Bruker TOPSPIN 2.0 software.

$^1$H NMR spectra were obtained at 400.16 MHz. The chemical shifts ($\delta$) were reported as ppm, and the residual solvent signal (4.80 ppm) was used as the internal reference. Induced changes in the $^1$H NMR chemical shifts ($\Delta\delta$) for trimethoprim and HP-β-CD originated due to their complexation were calculated according to the following equation:

$$\Delta\delta = \delta_{\text{complex}} - \delta_{\text{free}} \quad (1)$$

NMR spectra of pure components and their combinations were taken in $D_2O$. The concentration of components was 1.4 mM for trimethoprim and 7 mM for HP-β-CD.

### 2.5. Solid samples preparation

The preparation of a solid complex trimethoprim: HP-β-CD with 1:1 molar ratio was performed by the freeze-drying method.[19] Appropriate amounts of each component were suspended in water, and sonicated at 25.0 ± 0.1 °C constant water temperature until the drug was dissolved completely. Solutions were frozen at −40 °C for 24 h to ensure a complete solidification, before the freeze-drying was started (Freeze Dye 4.5 Labconco corp., Kansas City, MI).

Physical mixtures were prepared by mixing uniformly in a mortar the corresponding components with 1:1 molar ratio.

### 2.6. Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectra were recorded on a Nicolet 5 SXC FT-IR Spectrophotometer (Madison, WI, USA). The potassium bromide disks were prepared by compressing the powder.

### 2.7. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)

The DSC curves of the samples were performed with a DSC TA 2920 and the TGA curves were recorded on a TG TA 2920. The samples were placed in aluminum hermetic pans, and the experiments were carried out under a nitrogen gas flow, at a heating rate of 10 °C/min, over a temperature range of 25–400 °C.

### 2.8. Scanning electron microscopy (SEM)

Microscopic morphological structures of the raw materials, the binary complex, and the physical mixture were investigated and photographed using a scanning electron microscope LEO Model EVO 40XVP. The samples were fixed on a brass stub using a double-sided aluminum tape. To improve the conductivity, samples were gold-coated under vacuum employing a sputter coater PELCO Model 3. The magnification selected was sufficient to appreciate in detail the general morphology of the samples under study.

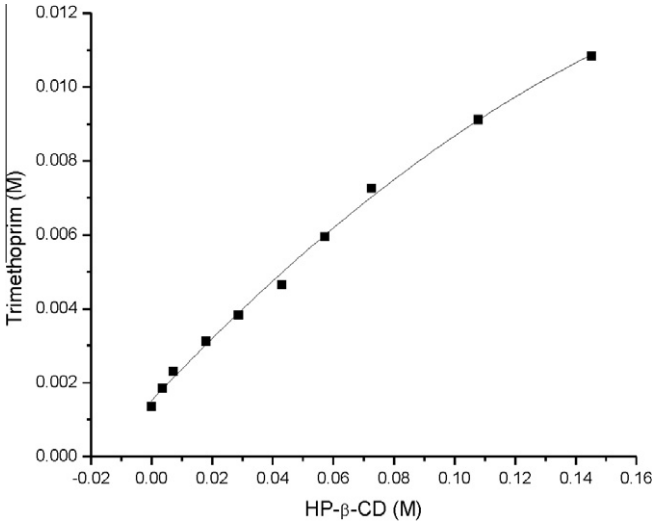


**Figure 3.** Effect of HP-β-CD on the solubility of trimethoprime in aqueous solution at 25.0 °C.

## 3. Results and discussion

### 3.1. Phase solubility analysis

The effect of HP-β-CD on the solubility of trimethoprim was investigated. The solubility of trimethoprim in water was determined to be 0.4 mg/ml. The solubility profile (Fig. 3) showed an increase in its solubility when the concentration of HP-β-CD increases. The solubility of trimethoprim was of 1.1 mg/ml, 2.1 mg/ml, and 3.2 mg/ml in the presence of 28.6, 71.4, and 142.9 mM HP-β-CD solution, respectively. Therefore, HP-β-CD increased the solubility of trimethoprim by approximately three, five, and eightfold, respectively, compared to those in aqueous solutions. These results suggest that HP-β-CD is an effective solubilizing agent for trimethoprim.

The solubility diagram with a negative deviation from linearity as a function of HP-β-CD concentration indicates that the solubilizer was less effective at higher concentrations. This curve, classified as $A_N$ type, may be associated with both an alteration in the effective nature of the solvent in the presence of large concentrations of HP-β-CD and a self-association of HP-β-CD at higher concentrations. These associations might affect the apparent degree of complexation.[18,20,21] These results were in agreement with the work of Pourmokhtar and Jacobson,[16] which described a non-linear complexation of trimethoprim with HP-β-CD in the range of concentrations between 2% and 15%.

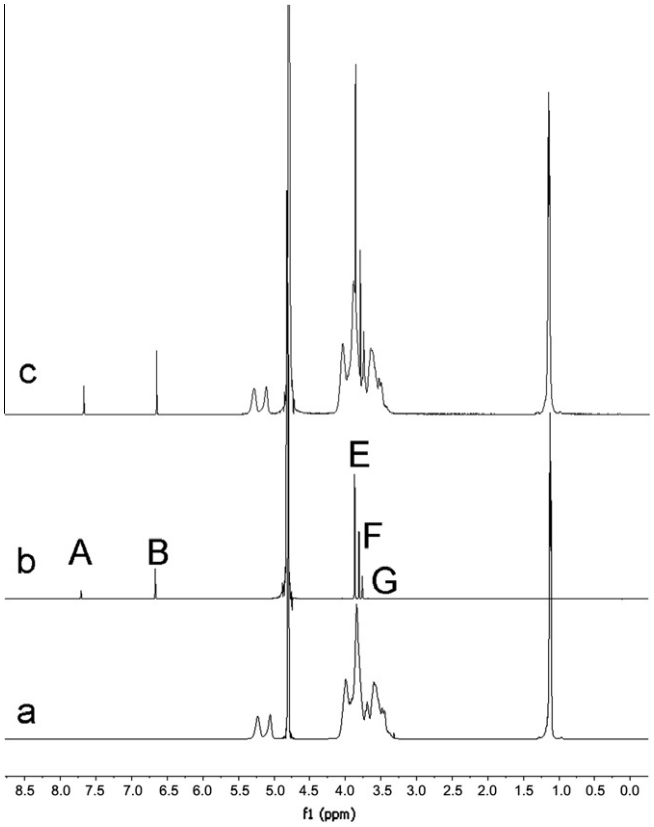


**Figure 5.** $^1$H NMR spectra in $D_2O$ of: (a) trimethoprim:HP-β-CD complex, (b) trimethoprim, (c) HP-β-CD.

#### 3.1.1. Evaluation of the apparent stability constant by phase solubility analysis

In all complexation processes, the measurement and knowledge of the stability constant are crucial since this value provides an index of change of physico-chemical properties that result upon host–guest binding. A CD complex with a 1:1 stoichiometry ratio (S:L) shows the following equilibrium:

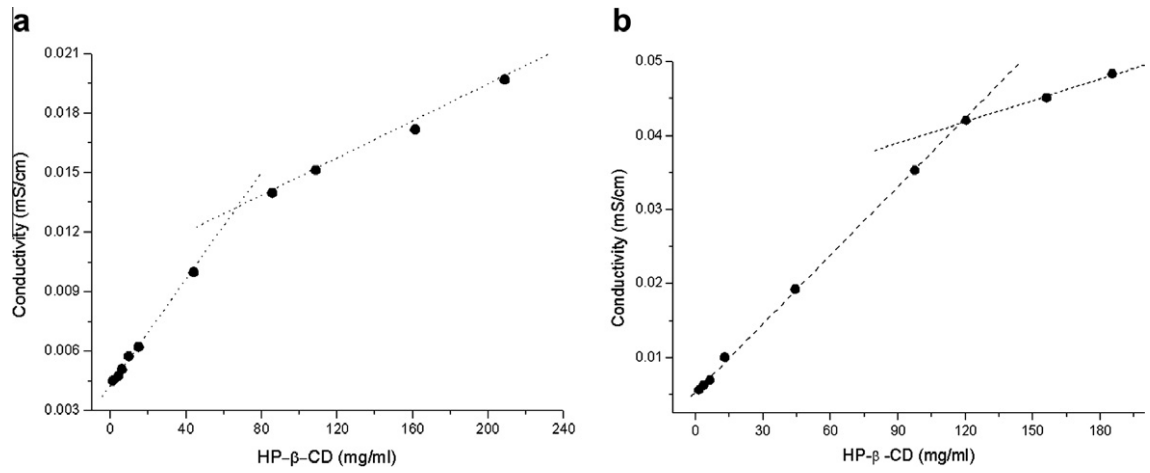


**Figure 4.** Conductivity measurements as a function of concentration for aqueous solutions of: (a) HP-β-CD and (b) trimethoprim:HP-β-CD, at 25 °C.

**Table 1**
$^1$H NMR chemical shifts ($\delta$) of the individual components and changes ($\Delta\delta$) in the presence of the binary system TMP:HP-β-CD

| | Trimethoprim | | | | HP-β-CD | | |
|---|---|---|---|---|---|---|---|
| | $\delta_0$[a] | $\delta_C$[b] | $\Delta\delta$[c] | | $\delta_0$[a] | $\delta_C$[b] | $\Delta\delta$[c] |
| A | 7.686 | 7.654 | −0.032 | H1 | 5.142 | 5.201 | 0.059 |
| B | 6.647 | 6.640 | −0.007 | H2 | 3.599 | 3.661 | 0.062 |
| E | 3.852 | 3.874 | 0.022 | H3 | 3.997 | 4.053 | 0.056 |
| F | 3.792 | 3.806 | 0.014 | H4 | 3.468 | 3.532 | 0.064 |
| G | 3.746 | 3.760 | 0.014 | H5 | 3.693 | — | — |
| | | | | H6 | 3.841 | 3.901 | 0.060 |
| | | | | $CH_3$ | 1.120 | 1.182 | 0.062 |

[a] $\delta_{free}$.
[b] $\delta_{complex}$.
[c] $\Delta\delta = \delta_C - \delta_0$.

$$S + L \overset{K_{1:1}}{\rightleftharpoons} S:L \quad (2)$$

where S is the substrate and L is the ligand. The apparent stability constant ($K_{1:1}$) for this equilibrium is defined as:

$$K_{1:1} = \frac{[S:L]}{[S][L]} \quad (3)$$

The assumption about obtaining a soluble inclusion complex trimethoprim:HP-β-CD which has a 1:1 stoichiometry was based upon the initial linear portion of the phase solubility diagram that showed an increase in the trimethoprim solubility with increasing the concentration of HP-β-CD at low concentrations of this oligosaccharide.

The $K_{1:1}$ value of 60.4 ± 0.2 $M^{-1}$ was estimated from the linear section of the solubility diagram (Fig. 3) and the solubility of trimethoprim in water ($S_0$) according to the following equation:

$$K_{1:1} = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (4)$$

Li et al.[14] and Mura et al.[12] have described the phase solubility studies of trimethoprim:β-CD complex, they reported $K_{1:1}$ values of 65 $M^{-1}$ and 82 $M^{-1}$, respectively, which are similar to the results obtained in this study. These results suggest that the solubilization process is the same between these CDs, regardless of the substitution of the external hydroxyl groups of the toroidal shape.

### 3.2. Aggregation behavior of HP-β-CD and trimethoprime:HP-β-CD complex

The CDs and their complexes show a tendency to self-associate by forming aggregates in aqueous solution. The formation of such aggregates and drug solubilization within these structures can explain the observed phase solubility diagrams.[22,23]

Conductivity was used to obtain further information on aggregates formation and critical concentrations for HP-β-CD and trimethoprim:HP-β-CD complex. In Figure 4 we show representative plots of conductivity as a function of the HP-β-CD concentration for aqueous solutions of both the host and the complex at 25 °C. We observed that the increase of the host concentration modified the mobility of the species in solution, and induced an increase in the conductivity.

The change in the slope of the plots demonstrated that at high HP-β-CD concentrations the aggregates formation is favored. The critical concentrations were determined from the intersection point of the linear segments, corresponding to the monomeric and aggregate form of the HP-β-CD. For the criterion of the fit the best correlation coefficient was chosen. The critical concentration for HP-β-CD was 69.3 mg/ml (about 49.5 mM). This value is in a reasonable agreement with the value (77.7 mg/ml) obtained previously by $^1$H NMR.[24] The differences between the determined values arise from the use of distinct methods of determination due to the fact that different techniques measure different physico-chemical parameters and that the critical concentration is a concentration range at which the aggregation of free monomers begins.[25]

On the other hand, the significant increases in the conductivity observed for the system trimethoprim:HP-β-CD can be due to the interaction between trimethoprim and HP-β-CD. The critical concentration value determined was 117.7 mg/ml (about 84.0 mM). These experimental results indicated that trimethoprim weakens the intermolecular forces that held together the HP-β-CD

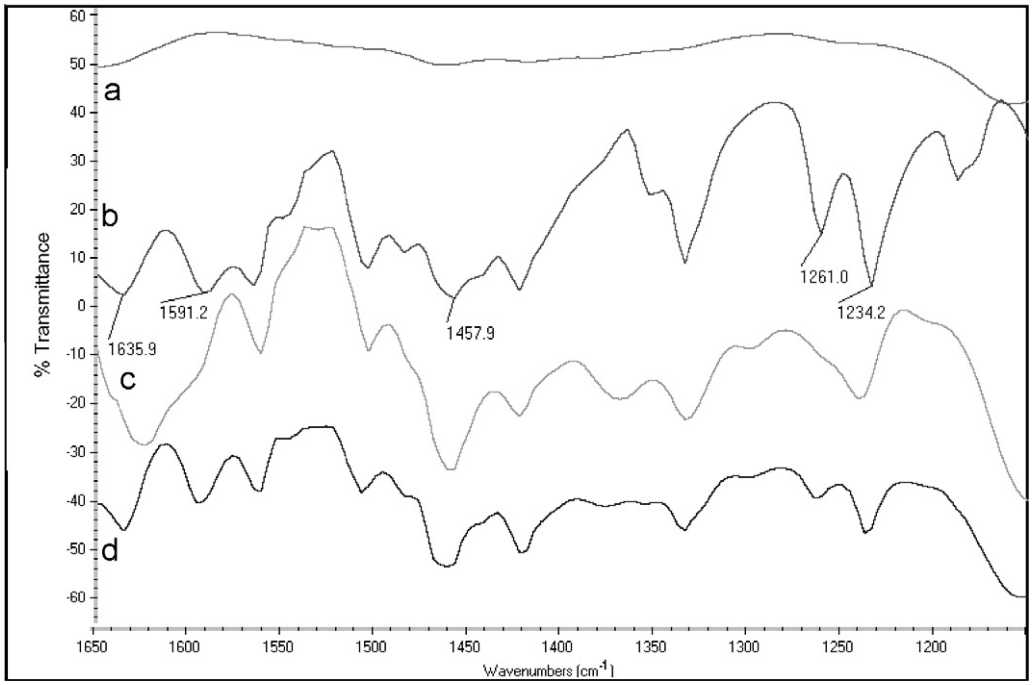


**Figure 6.** FT-IR spectra of: (a) HP-β-CD, (b) trimethoprim, (c) trimethoprim:HP-β-CD freeze-dried, (d) trimethoprim:HP-β-CD physical mixture.

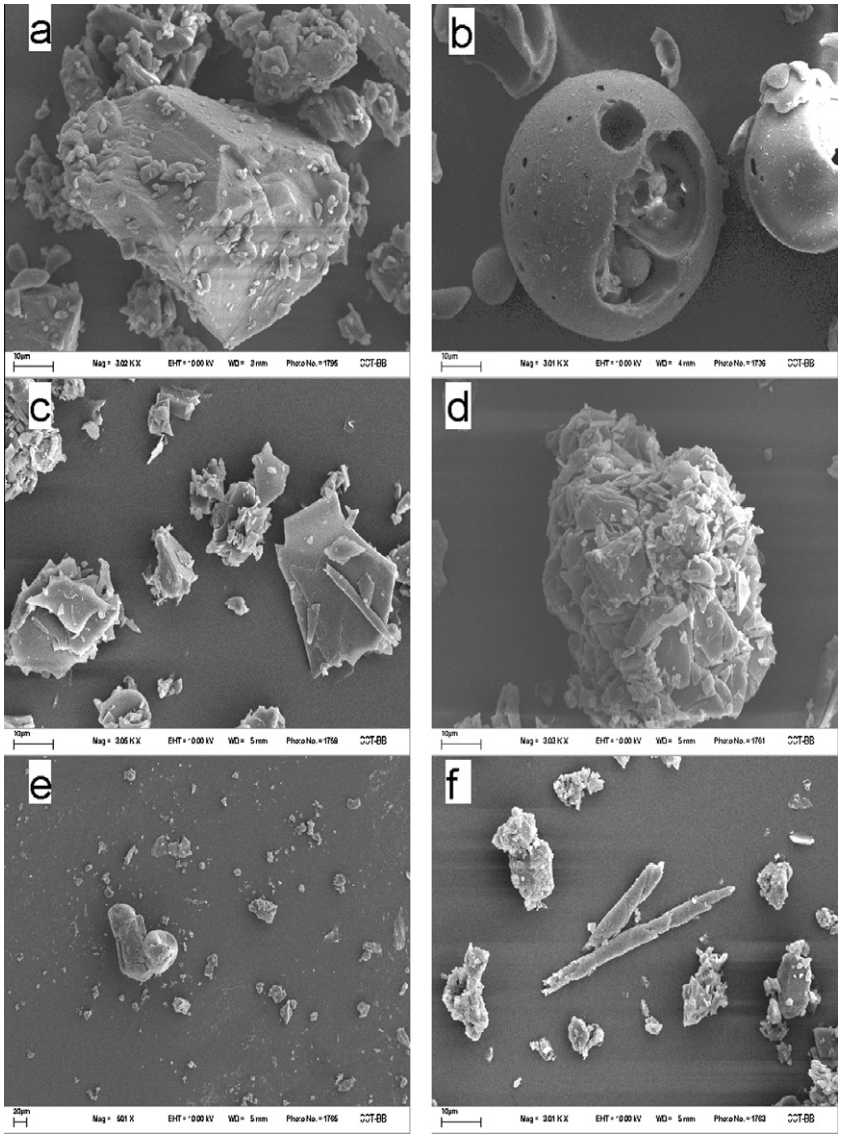


**Figure 7.** Scanning electron microphotographs of: (a) trimethoprim, (b) HP-β-CD, (c) and (d) trimethoprim:HP-β-CD freeze-dried, (e) and (f) trimethoprim:HP-β-CD physical mixture.

molecules in the aggregates, as suggested by the higher critical concentration value for the complex with respect to free HP-β-CD.[26] The self-association between trimethoprim:HP-β-CD complexes as well as between free HP-β-CD molecules and the complexes can explain the observed solubilization phenomena which have a negative deviation from linearity (Fig. 3).

### 3.3. Study by $^{1}$H nuclear magnetic resonance spectroscopy ($^{1}$H NMR)

NMR spectroscopy is the most powerful tool for the study of formation of inclusion complex between CDs and a variety of guest molecules, since the chemical and electronic environments of protons are affected during complexation, which are reflected through changes in the $\delta$. The inclusion of trimethoprim into the HP-β-CD cavity was firstly evaluated analyzing the changes observed in the $\delta$ of the protons in the complex in comparison to free trimethoprim and HP-β-CD (Fig. 5). Table 1 presents the assignment of trimethoprim and HP-β-CD protons and the $\Delta\delta$ originated by complexation.

Since there are no new peaks that could be assigned to the pure inclusion compound, complexation of trimethoprim with HP-β-CD appears to be a dynamic process with the trimethoprim being in a state of fast exchange (relative to the NMR timescale).

In presence of trimethoprim, appreciable downfield shifts for HP-β-CD protons which evidenced host–guest interactions were observed. In fact, these shifts could be the result of a deshielded effect, suggesting that the trimethoprim molecules produce paramagnetic anisotropy effects in the interior of the cavity due to weak interactions (van der Waals forces). The H-5 signal could not be directly observed, because it was overlapped with trimethoprim intense signals. The modifications observed for HP-β-CD protons indicated that the CD cavity was deformed as a consequence of interactions between the guest and the host molecules, and gave evidence of the existence of a partial or complete inclusion.

All trimethoprim protons showed shifts. A and B protons to upfields; and E, F, and G protons to downfields. Upfield shifts indicate that these protons are close to a host atom rich in π-electrons. In this case, such effects can be associated with oxygen atoms, and also can reflect conformational changes produced by the inclusion phenomena. The downfield shifts can be attributed to a variation of local polarity when these protons are inside the cavity or at a

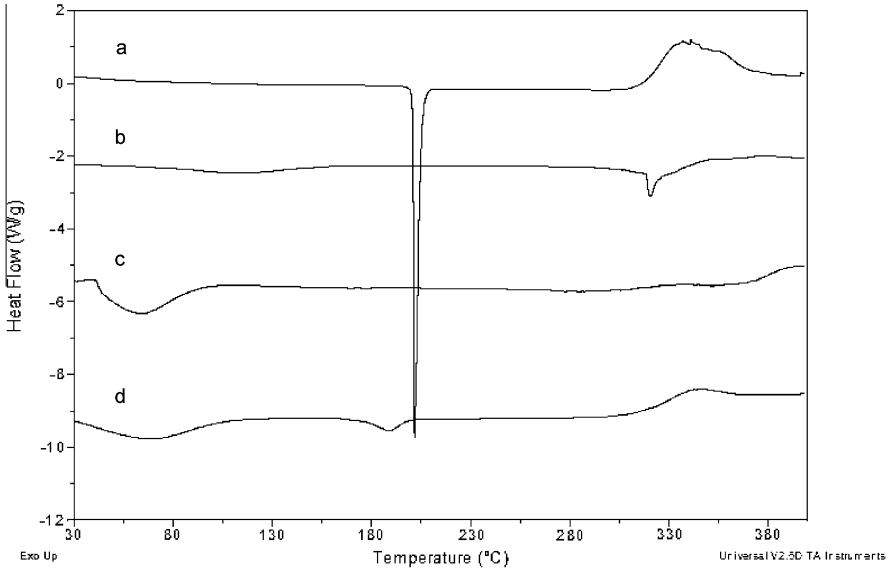


**Figure 8.** DSC curves of: (a) trimethoprim, (b) HP-β-CD, (c) trimethoprim:HP-β-CD freeze-dried, (d) trimethoprim:HP-β-CD physical mixture.

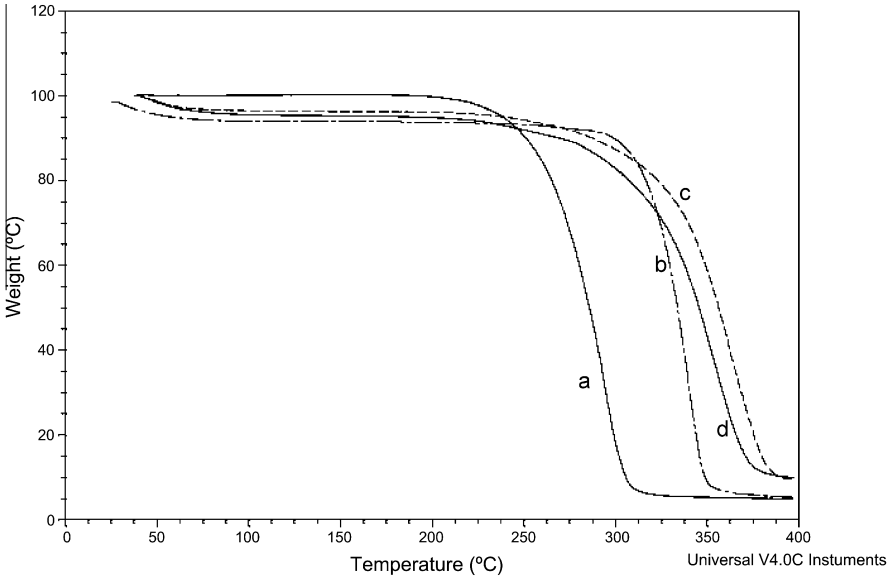


**Figure 9.** TGA curves of: (a) trimethoprim, (b) HP-β-CD, (c) trimethoprim:HP-β-CD freeze-dried, (d) trimethoprim:HP-β-CD physical mixture.

deshielding effect due to van der Waals forces between trimethoprim and the carbohydrate chains. These findings suggest the formation of an inclusion complex between trimethoprim and HP-β-CD.

### 3.4. Solid-state studies

Some information on solid-state interactions between trimethoprim and HP-β-CD was obtained by FT-IR, SEM, DSC, and TGA.

#### 3.4.1. Fourier-transform infrared spectroscopy (FT-IR)

FT-IR spectroscopy has been used to assess the interaction between CD and guest molecules in the solid state due to the fact that complexation of the guest produces shifts or changes in the absorption spectrum. The FT-IR spectra of trimethoprim, HP-β-CD, physical mixture, and freeze-dried system are shown in Figure 6. FT-IR spectrum of trimethoprim showed characteristic bands at 1635.9 and 1591.2 $cm^{-1}$ (combination of deformation of $NH_2$ group and stretching of aromatic ring), 1457.9 $cm^{-1}$ (deformation of $CH_2$ group), 1261.0 $cm^{-1}$ (vibrations of C–N in the amine aromatic primary), and 1234.2 $cm^{-1}$ ($OCH_3$ aromatic group vibrations). The bands originated due to $NH_2$ and C–H aromatic stretching vibrations could not be analyzed because their overlap with the band attributed to O–H stretching vibrations of HP-β-CD.

The trimethoprim: HP-β-CD freeze-dried system spectrum did not show new bands, indicating that no chemical bonds were created in the formed complex. Though, it showed that the bands assigned to the combination of deformation of $NH_2$ group and stretching of aromatic ring changed to a unique band at 1625.2 $cm^{-1}$. This change shows strong interactions between trimethoprim and HP-β-CD. The band assigned to $CH_2$ group increased its intensity and shifted, the characteristic C–N band disappeared, and the band of the methoxy aromatic group was broader and shifted to higher frequency. These changes suggest the formation of an inclusion complex between trimethoprim and HP-β-CD in solid state. On the other hand, the spectrum of

the physical mixture corresponded simply to the superposition of the FT-IR spectra of the two components, suggesting absence of interactions.

### 3.4.2. Scanning electron microscopy (SEM)

SEM is a qualitative method used to study the structural aspects of raw materials, and the products obtained by different methods of preparation.[27] Supporting evidence for the complexation of trimethoprim with HP-β-CD was also obtained from SEM. Figure 7 illustrates the SEM microphotographs of pure materials, physical mixture, and freeze-dried system at different magnifications.

Typical crystals of trimethoprim were seen as a compact structure with irregular borders. Those crystals were found in many different sizes and shapes. The smaller particles are adhered to the surfaces of the larger ones. Whereas, in the HP-β-CD microphotographs, hollow spherical particles with a broad size distribution (10–50 μm) were evident. In addition, it was observed that large particles contain their interior particles of minor size, which can be assumed as an HP-β-CD aggregation in the solid powder.

A drastic change in the morphology and shape of particles was observed in the freeze-dried product. Microphotographs showed the presence of amorphous particles of irregular size and shape, in which the original morphology of both components disappeared indicating the interaction of the drug with HP-β-CD in solid state. At high magnification, it was possible to distinguish compact and amorphous arrangements formed by particles with smooth surfaces and irregular borders, which can be attributed to the presence of aggregates in the system. On the contrary, in SEM pictures of physical mixture, the characteristic trimethoprim crystals, which were mixed with HP-β-CD particles, were clearly detectable, confirming the presence of crystalline drug and absence of interaction in this solid system. HP-β-CD particles lost their original shape and in this case sizes were smaller. This modification can be attributed to the mixing process of the solid one.

SEM pictures demonstrated the difference in the morphology of the systems, obtained by freeze-drying method and by physical mixing. The drastic change in the morphology of lyophilized system revealed a solid-state interaction and constitutes a clear evidence of a new solid phase formation resultant of the molecular complexation of trimethoprim in the cavity of the HP-β-CD.

### 3.4.3. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)

Thermal analysis has been reported as a method to characterize CD complexes.[28] It provides additional evidence when guest molecules are embedded in CD cavities or in the crystal lattice. The DSC and TGA profiles of trimethoprim, HP-β-CD, physical mixture, and freeze-dried system are shown in Figures 8 and 9, respectively.

Trimethoprim showed a sharp endothermic peak at 201.77 °C, due to drug melting followed by a broad exothermic peak due to decomposition phenomena associated with a loss of the mass fraction of 95% beginning at 266 °C. HP-β-CD exhibited a typical broad endothermic peak between 50 and 175 °C, assigned to a dehydration process (6.5% mass loss) that corresponds to a loss of about 4.5 water molecules per HP-β-CD molecule. Also, up to 300 °C, the CD decomposition begins to appear.

The DCS curve of the binary system prepared by the freeze-dried method showed the complete disappearance of the trimethoprim peak, indicating the molecular encapsulation of the drug inside the CD cavity. Interestingly, the TGA curves for the system showed that the dehydration stage contains only 3.7% of water in relation to the 4.6% of the physical mixture. This indicated that most of the water molecules in the HP-β-CD cavity are replaced by trimethoprim during the inclusion process. In addition, the decomposition of trimethoprim started above 329 °C, a temperature that is considerably higher than that of the pure drug (266 °C), suggesting the considerable enhancement of the thermal stability of trimethoprim in solid state by the formation of the inclusion complex. Finally, in the physical mixture, the characteristic events observed for the individual curves of trimethoprim and HP-β-CD were found. These patterns were in agreement with IR spectroscopic data regarding the formation of the complex.

## 4. Conclusion

In conclusion, the results reported here revealed that the solubility of trimethoprim was enhanced in presence of HP-β-CD. At low HP-β-CD concentrations, the formation of an inclusion complex produced an increase in the solubility; whereas at concentrations higher than the critical concentration, aggregates capable of solubilizing trimethoprim through the formation of non-inclusion complexes were formed.

## Acknowledgments

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