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Preparation and characterization of Trimethoprim inclusion complex with Methyl- β -Cyclodextrin and determination of its antimicrobial activity

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Abstract

The present study focuses on the formation and characterization of inclusion complexes between Trimethoprim (TMP), an inhibitor of bacterial dihydrofolate reductase, and cyclodextrins, namely, methyl-βeta-cyclodextrin (MBCD) and hydroxylpropyl-βeta-cyclodextrin (HBCD) in aqueous solution. MBCD was selected to prepare inclusion complexes in the solid state. These complexes were prepared by different methods: spray drying, kneading and freeze drying. Physical mixtures were prepared as reference. The prepared systems were then characterized by different techniques: Differential scanning calorimetry (DSC), Fourier Transform Infrared Spectroscopy (FT-IR) and Scanning Electron Microscopy (SEM). The dissolution profile and the antimicrobial activity of Trimethoprim and the inclusion complexes were evaluated using the dissolution test, and the disk diffusion methodology, respectively.

An increase of TMP solubility was observed in phase solubility studies. The obtained apparent stability constants (Ks) showed that MBCD formed an inclusion complex more stable with the drug than HPBCD, so it was decided to prepare inclusion complexes in solid state with MBCD. The results obtained with DSC, FTIR and SEM proved the formation of inclusion complexes in solid state. The dissolution profile and the antibacterial activity increased with the complexation process.

Keywords: Cyclodextrins; Trimethoprim; Inclusion complexes; Antibacterial activity

Abbreviation: BCS: Biopharmaceutical Classification System; CLSI: Clinical and Laboratory Standards Institute; CDs: cyclodextrins; CGTase: Cyclodextrin glycosyl transferase; DSC: Differential Scanning Calorimetry; FD: Freeze Dried System; FT-IR: Fourier Transform Infrared Spectroscopy; HBCD: Hydroxylpropyl-βeta-cyclodextrin; HCl: Hydrochloric acid; KN: Kneaded System; Ks: Apparent Stability Constants; MBCD: Methyl-βeta-cyclodextrin; MW: Molecular weight; NADPH: Nicotinamide adenine dinucleotide phosphate; PM: Physical Mixture; S_0: Intrinsic Solubility; SD: Spray Dried System; SEM: Scanning Electron Microscopy; S_f: Final solubility in the presence of cyclodextrins; TMP: Trimethoprim; α: Alpha; β: Beta; γ: Gama

Introduction

TMP (5-(3,4,5-trimethoxybenzyl)-pyrimidine-2,4-diamine), (Figure 1) is a synthetic antibiotic, which inhibits bacterial dihydrofolate reductase, the enzyme that reduces the dihydrofolate to tetrahydrofolate together with the cofactor nicotinamide adenine dinucleotide phosphate (NADPH), thus inhibiting the synthesis of nitrogenous bases necessary for the replication of bacterial nucleic acids [1].

This drug is used in the treatment of uncomplicated urinary tract infections caused by aerobic Gram-positive cocci (*Streptococcus pneumoniae*, *Staphylococcus aureus*) and coagulase-negative species, including *Staphylococcus saprophyticus* and also shows activity towards Gram-negative organisms (*Enterobacter spp*., *Escherichia coli*, *Haemophilus influenzae*, *Salmonella spp*., *Proteus mirabilis*, *Serratia spp*., *Providencia spp*., *Klebsiella spp*., *Proteus mirabilis*, *Shigella spp*., *Morganella morganii*) [1].

According to the Biopharmaceutical Classification System (BCS), TMP is classified as a class II drug showing low solubility and high permeability due to its lipophilicity, which reduces its bioavailability [2].

A strategy used to increase the solubility, and bioavailability of poorly water soluble compounds such as TMP is the formation of inclusion complexes with cyclodextrins (CDs) [3].

CDs are oligosaccharides which are formed by D-(+) glucopyranose units linked with alpha-1,4-glycosidic bonds and are obtained by enzymatic degradation of the starch by action of the enzyme cyclodextrin glycosyl transferase (CGTase) [4]. The natural cyclodextrins obtained with higher yield by the action of the CGTase are α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin that contain six, seven and eight glucose units, respectively. Due their physicochemical properties, their structure with an hydrophilic internal cavity and an hydrophobic exterior, they can be used as host for a variety of guest molecules to form inclusion complexes by non-covalent bonds.

Despite the fact that the natural CDs are widely used for the investigation and development of pharmaceutical formulations, they have some limitations. In particular, β-cyclodextrin shows a low aqueous solubility (1.85% w/v at 25°C) due to its rigid structure resulting from intramolecular formation of hydrogen bonds between its secondary hydroxyl groups [4]. In order to counteract the low solubility of this cyclodextrin, chemically modified derivatives with higher solubility and lower toxicity have been developed, such as 2,3,6

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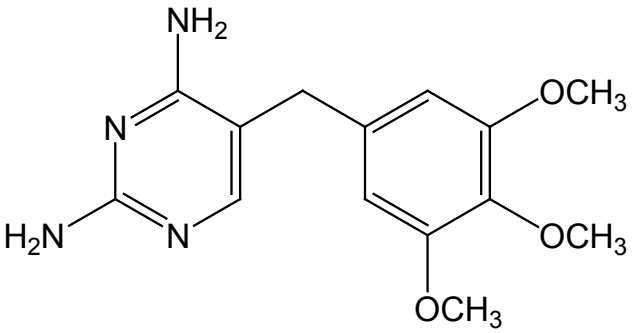

Figure 1: TMP molecular structure.

methyl–β-cyclodextrin (MBCD), randomly methylated in position 2, 3 and 6 hydroxylpropyl-β-cyclodextrin (HPBCD), formed by substitution of the hydroxyl in position 2, 3 and 6 with 2-hydroxypropyl substituents [4,5]

The complexation of TMP with MBCD and HPBCD in aqueous solution was also studied by other authors [6,7]. However, the full characterization of the formed complexes in solid state, including their dissolution profile and antibacterial activity determination was never performed.

Materials and Methods

Chemicals and reagents

Hydroxylpropyl-β-cyclodextrin (HPBCD, MW=1300) and Methyl-β-cyclodextrin (MBCD, MW=1190) were buy from Sigma Aldrich (Madrid, Spain); Trimethoprim (MW=290.3) was buy from JMSS (Odivelas, Portugal). All other reagents were of the highest purity available from commercial sources.

Phase solubility studies

The preparation of inclusion complexes in solution was performed according to the phase solubility method of Higuchi and Connors [8]. An excess of TMP was suspended in aqueous solutions containing increasing concentrations of HPBCD or MBCD (5 to 49 mM). The intrinsic solubility of TMP was determinate using the aqueous preparation in absence of CDs. The suspensions were stirred during 72 hours, protected from light until the equilibrium was reached. The excess of TMP was removed by filtration through a 0.45 μm membrane filter (Millipore). Clear solutions were suitably diluted and analyzed by UV-vis spectrophotometry (UV-1800 Spectrophotometry SHIMADZU), measuring the absorbance at 280 nm [9,10]. Apparent stability constants (Ks) were calculated from the phase solubility diagrams using the equation (1), where S_0 is the TMP solubility in the absence of MBCD or HPBCD (intercept) [11-13].

$$Ks = slope/S_0 (1 - slope)$$

Preparation of inclusion complexes in the solid state

Solid inclusion complexes were prepared according with phase solubility studies, by three different methods: kneading, freeze-drying and spray-drying. The physical mixture (PM) was prepared as a reference for comparison with the inclusion complexes.

Physical binary mixture (PM): TMP: MBCD PM was prepared by simply blending TMP and MBCD, previously sieved, with 1:1 molar ration in a mortar.

Kneaded binary system (KN): MBCD (0.8198 g) was added in a ceramic mortar with deionized water (20% of the final weight of the product) until a paste was obtained. The required amount of TMP (0.2 g) was added slowly and the slurry was kneaded. The final product was allowed to equilibrate during 72h at room temperature and humidity, protected from light.

Freeze-dried binary system (FD): MBCD (0.8198 g) was dissolved in 200 mL of aqueous solution under magnetic stirring. Then TMP (0.2 g) was added and the solution stirring was maintained for 72 hours. In order to obtain a clear solution, it was measured the pH and the solution was acidified with 1M HCl until reaching pH=3.

The resultant solution was frozen by immersion in an ethanol bath at –50°C (Labconco Shell Freezer) and, lyophilized (Labconco Freeze-Dryer) for 72 hours.

Spray-dried binary system (SD): The same procedure to prepare freeze-dried binary system was adopted to prepare spray-dried system. The resultant clear solution was subjected to spray drying (BUCHI Mini Spray Dryer B-290) using the following work conditions: Temperature inlet: 100°C; Temperature outlet: 37°C; Pump: 30mL/h; compressor (airflow rate): 40 m^3/L.

Differential scanning calorimetry (DSC)

Thermal analyses of the samples were carried out using Differential Scanning Calorimetry (DSC-60 SHIMADZU Differential Scanning Calorimeter). For this purpose, the thermal behavior was studied by heating the samples (2 mg) in a sealed aluminum pan from 25-250°C, at a rate of 10°C/min and under a nitrogen flow of 20cm^3/min, and using an empty pan sealed as reference [14]. Indium (99.98%, mp 156.65°C, Aldrich®, Milwaukee, USA) was used as standard for calibrating the temperature.

Fourier transform-infrared spectroscopy (FT-IR)

Spectra were recorded using a Jasco FT/IR-420 spectrometer associated with an ATR horizontal reflexion (Miracle™, PIKE Technologies). Spectra acquisitions were performed directly in powder samples with the application of 16 scans at a resolution of 4 cm^{-1} over the range 4000-400 cm^{-1}.

Scanning electron microscopy (SEM)

Morphological structures of the raw materials, the PM and the inclusion complexes were investigated and photographed using a Scanning Electron Microscope (JEOL JSM-6010LV). The samples were fixed on a brass stub using double-sided tape and then made electrically conductive by coating in vacuum with a thin layer of gold [15]. Photographs were taken with an excitation voltage of 5 and 10 KV and magnifications factors of 1000 and 2500.

Dissolution studies

In vitro dissolution studies were conducted in 1000 mL of distilled water using the USP2 apparatus [16,17], by adding the equivalent of 100mg of TMP for each test. The medium was previously filtered, degassed and maintained at 37 ± 0.5°C according to Portuguese Pharmacopeia [18].

The studies were conducted at 37°C and at a stirring speed of 100 rpm. Aliquots from samples were withdrawn each 15 min for a period of 75 min, filtered with a membrane filter (0.45 μm), and analyzed by UV–vis spectroscopy at 280 nm. Six replicates have been made for each experience.

Antibacterial activity determination

Three clinical isolated bacteria, two *Escherichia coli* and one *Staphylococcus aureus* from urinary tract infection were utilized for the determination of the antibacterial activity of TMP complexed with MBCD by different methods [19].

Susceptibility testing was performed by disk diffusion methodology, according to Clinical and Laboratory Standards Institute (CLSI) [20]. Guidelines, using TMP at concentration of 5μg/mL and binary systems at concentration of 40mg/mL, in order to obtain always the same concentration of TMP. Results were interpreted according to CLSI breakpoints [20].

Results

Phase solubility studies

Phase solubility studies of TMP with HPBCD and MBCD are shown in Figure 2. TMP solubility increased linearly with cyclodextrins concentration and the slope was smaller than unity, indicating an A_L type diagram with the formation of a complex with 1:1 stoichiometry for both cyclodextrins. The calculated K_S values for the inclusion complexes, the TMP aqueous solubility (S_0), the TMP solubility in cyclodextrin solutions (S_f) and the corresponding slopes are presented in Table 1. The TMP solubility increased, approximately, 2.91 and 3.58-fold in the presence of HPBCD and MBCD, respectively, comparing with S_0. The determinated K_S values of the inclusion complexes were 48.57 and 67.65 M^{-1} for HPBCD and MBCD, respectively, suggesting that the methylated derivate forms a more stable inclusion complex with TMP than HPBCD, so it was decided to prepare inclusion complexes in solid state using MBCD.

Differential scanning calorimetry (DSC)

Thermograms of the different samples are presented in Figure 3. The thermal curve of TMP shows an endothermic peak at 202.21°C, corresponding to the melting point of the drug [21].

The DSC curve of MBCD exhibits a typical broad effect between 30 and 100°C associated with crystal water losses [22]. Considering the PM system, it is possible to observe a broadening and a shift to lower temperatures of the drug melting point (170°C), which could be ascribed to some drug–cyclodextrin interaction. The disappearance of the TMP melting point in the complexed systems (KN, SD and FD) suggests the formation of a true inclusion complex indicating a more stable and strong interaction between drug and cyclodextrin in these systems [22].

Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectroscopy was used to observe the interactions between MBCD and TMP in the solid state, due to the fact that complexation changes the absorption spectrum of the drug.

The Figure 4 shows the absorption spectra of TMP, MBCD, PM and the inclusion complexes. The FTIR spectrum of TMP shows bands between 1653.34 and 1421.08 cm^{-1} that can be assigned to C=C and C=N stretching of the aromatic ring; 1234.58 cm^{-1} corresponding to the OCH_3 aromatic group vibration; vibration of the C-N bond at 1336.77cm^{-1} in the aromatic primary amine, and NH_2 stretching at 3468.39 and 3403.64 cm^{-1} [7]. In the MBCD spectrum it is observed a broad band at 3368.18 cm^{-1} corresponding to residues of OH methylated groups, and a strong band at about 1030.82 cm^{-1} attributed to the OCH_3 group. Spectra of the binary systems do not show new bands, indicating the absence of new bonds. In the region above 2500 cm^{-1} a wider band is observed probably due to a superposition of spectral features of the OH stretching of the CD and the NH_2 stretching of the TMP [7]. Significant changes are observed in the area between 1600 and 1400 cm^{-1} assigned to C=C aromatic stretching in free TMP due to the interaction between this region of the drug and cyclodextrin cavity [7] and an enlargement of the bands at 1030 cm^{-1} of the MBCD is observed due to the establish of interaction during complexes formation.

Scanning electron microscopy (SEM)

SEM is a qualitative method used to study the structural aspect of raw materials and the products obtained by complexation with cyclodextrins. Figure 5 illustrates the SEM microphotographs of raw materials, the PM and the inclusion complexes. Typical crystals of TMP are observed as a compact structure with irregular borders. Those crystals are found in many different sizes and shapes and the smaller particles are adhered to the surfaces of the largest ones [6].

The MBCD microphotograph shows amorphous spherical particles with a broad size distribution and it is observed that large particles contain particles of minor size inside, which can be assumed as an aggregation phenomenon in the solid powder [6]. In the PM system the characteristic TMP crystals which are mixed with MBCD particles are clearly detectable confirming the presence of crystalline drug and the absence of interactions in this solid system. However, MBCD particles lost their original shape and the sizes are smaller. This modification can be attributed to the mixing process. In the complexed systems are observed 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 MBCD in the solid state. The SD system shows amorphous and homogeneous

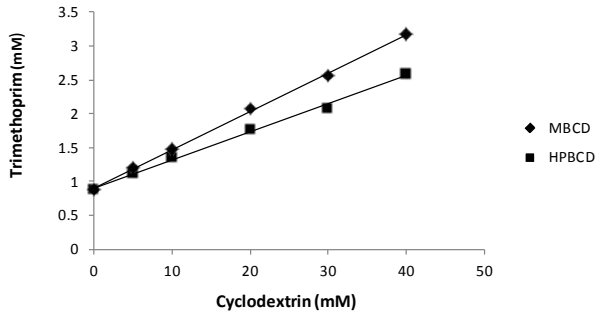

Figure 2: Phase solubility diagrams of TMP with CDs, in aqueous solution at 25±2°C. Values are mean±SD (n=3).

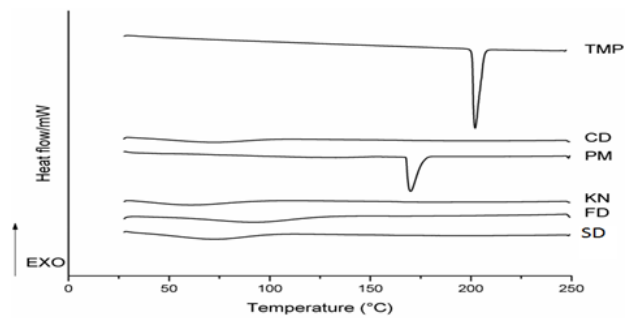

Figure 3: DSC curves of trimethoprim (TMP), methyl-beta-cyclodextrin (MBCD), physical mixture (PM), kneaded (KN), freeze-dried (FD) and spray-dried (SD) systems

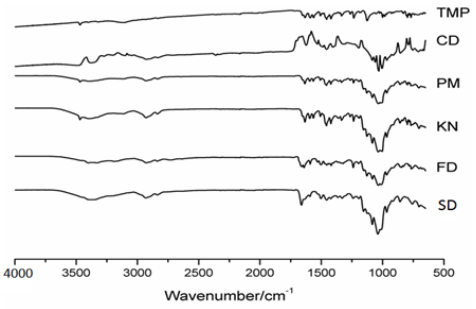

Figure 4: The infrared spectra of trimethoprim (TMP), methyl-beta-cyclodextrin (MBCD), physical mixture (PM), kneaded (KN), freeze-dried (FD) and spray-dried (SD) systems.

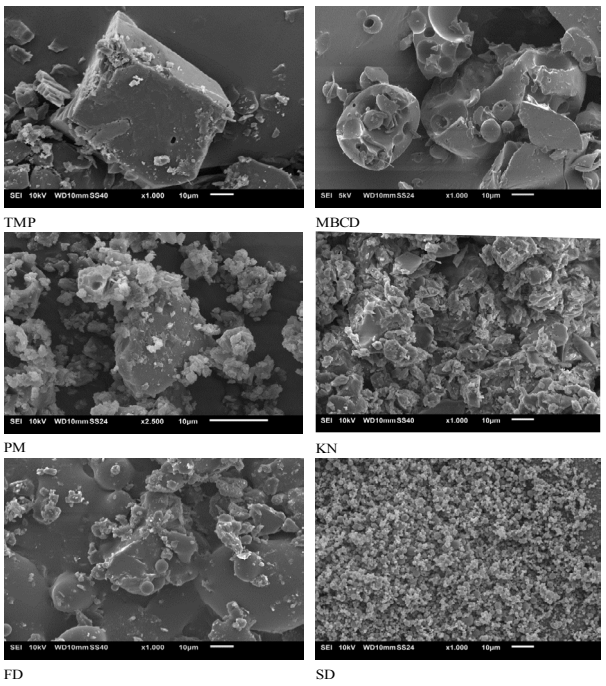

Figure 5: Scanning electron microphotographs of trimethoprim (TMP), methyl-beta-cyclodextrin (MBCD), physical mixture (PM), kneaded (KN), freeze-dried (FD) and spray-dried (SD) systems.

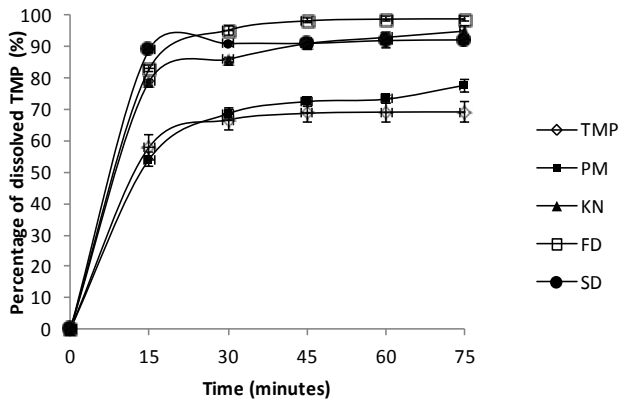

Figure 6: Dissolution profiles of trimethoprim (TMP), physical mixture (PM), kneaded (KN), freeze-dried (FD) and spray-dried (SD) systems.

aggregates of spherical particles, a particular aspect characteristic of this type of system [15].

Dissolution studies

The dissolution profiles of the TMP, the PM, and of the inclusion complexes are shown in Figure 6. The reported values are arithmetic means of six measurements. The release rate profiles are drawn as the percentage of drug dissolved vs time.

Table 2 shows the percentage of dissolved drug at the end of 15, 30, 45, 60 and 75 minutes. The data demonstrate that all binary systems show better dissolution properties compared to the TMP alone. After 75 minutes a high percentage of dissolution it is observed, especially for the FD system, which showed 99% of dissolved drug. These results suggest that complexation with MBCD can increase the efficacy of dissolution of TMP.

Antibacterial activity determination

The purpose of the antibacterial activity assay is to evaluate the microbiological properties of the complexes in order to study if the therapeutic effects of TMP are preserved despite complexation with the MBCD.

Table 3 shows the measurement data of the diameters of inhibition obtained from the TMP, PM and inclusion complexes, and taking as reference the CLSI breakpoint of TMP for *E. coli* and *S. aureus* [20]. It is possible to observe that inclusion complexes retain the antibacterial ability against *E.coli* R and *S. aureus* and also that the complexes obtained by freeze-drying and spray-drying methods increase the surface area of inhibition (Figure 7).

Inclusion complex	slope	S_0 (mM)	S_f (mM)	K_s (M^{-1})
TMP:HPBCD	0.0413	0.8875	2.5901	48.57
TMP:MBCD	0.0566	0.8875	3.1790	67.65

Table 1. Slope, S_o, S_f and K_s values for the inclusion complexes.

Time	Percentage (%)				
	TMP	PM	KN	FD	SD
0	0	0	0	0	0
15	58	54	79	83	89
30	67	69	86	95	91
45	69	73	91	98	91
60	69	73	93	99	92
75	69	78	95	99	92

Table 2. Percentage of drug dissolved in function of the time for the following systems: trimethoprim (TMP), physical mixture (PM), kneaded (KN), freeze-dried (FD) and spray-dried (SD).

Sample	Zone of inhibition in millimeters (mm)		
	E.coli S.	*E. coli R*	*S. aureus*
TMP	18.5	29.6	22.5
PM	16.0	28.5	20.2
KN	17.0	29.0	19.5
FD	14.0	30.3	26.0
SD	14.0	30.7	26.7

Table 3. Antibacterial activity of trimethoprim (TMP), physical mixture (PM), kneaded (KN), freeze-dried (FD) and spray-dried (SD) systems against bacterial test organism.

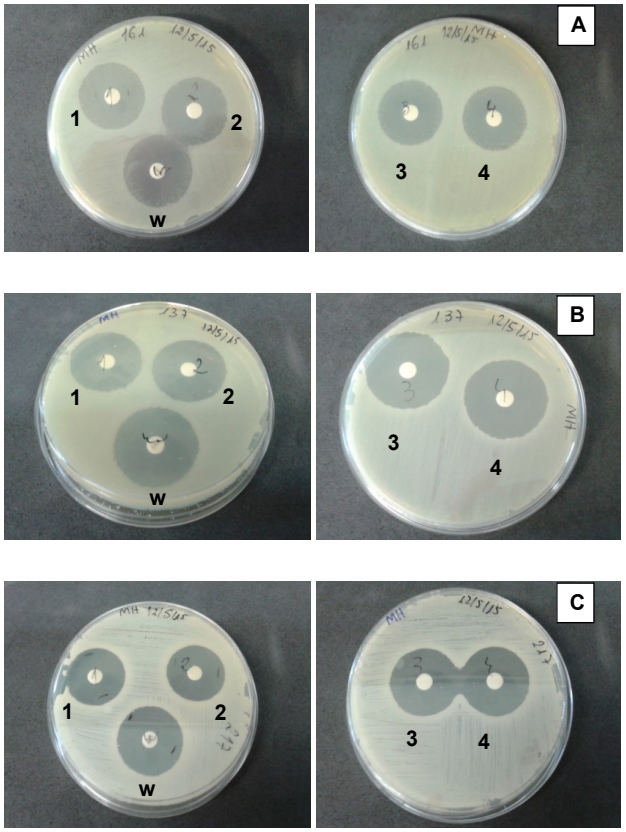

Figure 7: Antibacterial activity of: w) trimethoprim (TMP), 1) physical mixture (PM), 2) kneaded (KN), 3) spray-dried (SD) and 4) freeze-dried (FD) systems. (A) *Escherichia coli S.*; (B) *Escherichia coli R.* (C) *Staphylococcus aureus*.

Conclusion

The results of the phase solubility studies reveal that the solubility of TMP increases in the presence of MBCD and HPBCD. However the calculated apparent stability constants allowed concluding that TMP forms more stable inclusion complexes with MBCD than those formed with HPBCD.

The data obtained from DSC, FTIR and SEM show that a stable TMP:MBCD inclusion complex can be prepared at a 1:1 molar ratio by spray-drying and freeze-drying methods with a good performance of dissolution profile. The study of antibacterial activity shows that the inclusion complexes can retain the antibacterial ability of TMP and the complexes formed by spray-drying and freeze-drying methods can improve TMP therapeutic activity.

Taking into account these results, it is possible to conclude that the complexation effectively enhances the solubility of TMP, which consequently can increase its bioavailability and might improve its pharmaceutical potential.

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