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Original Research article

Effect of Trimethoprim Inclusion Complexation with Cyclodextrins on its Antimicrobial Activity

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ABSTRACT

The aim of present study is to highlight the effects of *β*-cyclodextrin (BCD) and hydroxypropyl-*β*-cyclodextrin (HBCD) and also the effect of their concentrations and methods of inclusion complexation on solubility and antibacterial activity of trimethoprim [TMP] by inclusion complex formation. The inclusion complexes of TMP were prepared by solvent evaporation, spray drying, kneading and physical mixture methods in 1:1 and 1:2 ratios. The inclusion complexes were characterized by Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), differential scanning calorimetry (DSC), dissolution study and antimicrobial activity by disk diffusion method.

Fourier transform infrared spectroscopy (FT-IR) and X-ray diffraction (XRD) results proved formation of inclusion complex of TEM with cyclodextrins. XRD showed decrease in crystanality of TEM after complexation with CDs. The results of saturation solubility study and release study prevailed the more increase in solubility of TMP by HPCD than BCD. The antibacterial activity of TMP was found to be increased with the complexation process. An increase in concentration of CD increased the dissolution and the antibacterial activity.

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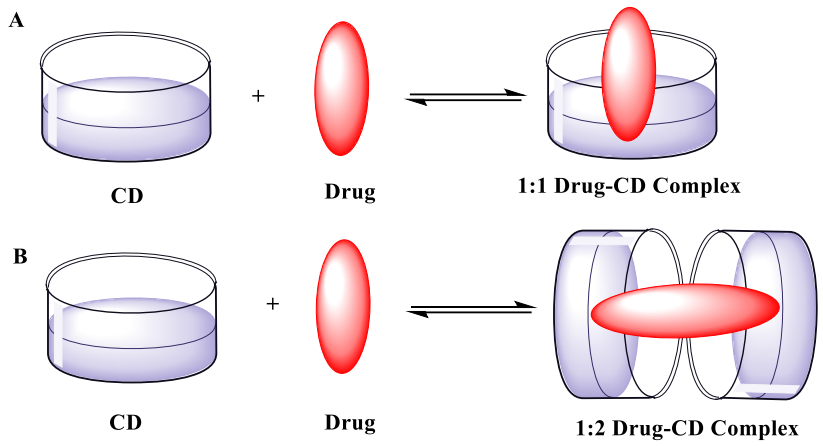
Graphical Abstract



Introduction

Trimethoprim (TMP) [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] is a bacteriostatic antibiotic used mainly in the prevention and treatment of urinary tract infections. TMP is a type of chemotherapeutic agent known as dihydrofolate reductase inhibitors. Trimethoprim inhibits the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF) by binding to dihydrofolate reductase [1].

Trimethoprim (TMP) shows activity against gram-positive organisms like *Streptococcus pneumoniae*, *Staphylococcus aureus* and also shows activity towards gram-negative organisms such as *Enterobacter spp.*, *Escherichia coli*, *Haemophilus influenzae*, *Salmonella spp.*, *Proteus mirabilis* [2].

Trimethoprim (TMP) shows low solubility and high permeability as it is BCS class II drug according to the biopharmaceutical classification system. TMP possess poor solubility in water; that is, 400 mg L^{-1} (≈ 1.377 × 10^{-3} M). Hence, there is a need in solubility enhancement of TMP. The chemical structure of TMP is shown in Figure 1.

Cyclodextrins (CDs) are chemical entities of natural origin, which are derived from bacterial degradation of starch through the metabolic action of cyclodextrin glycosyl transferase enzyme (GCTase). CDs have lipophilic inner cavities and hydrophilic outer surfaces are capable of interacting with a large variety of guest molecules. Chemically, they are cyclic oligosaccharides containing at least 6 D-(+)glucopyranose units attached by α-(1,4)glucosidic bonds. The general structure of CDs is based on D-glucopyranose units linked 1, 4, as in amylose. Three ring-types are common, where alpha-cyclodextrin is composed of six units, β-cyclodextrin of seven, and gamma-cyclodextrin of eight glucose units (CD) [3].

The objectives of present study are

1. Enhancement of solubility of TMP by CD-inclusion complexation.
2. To study effect of CD on antimicrobial activity of TMP.
3. To study effect of concentration of CD on dissolution and antimicrobial activity of TMP.

Experimental

Materials

Trimethoprim (TMP) the model drug was procured from Rajesh chemicals, Mumbai.

β-cyclodextrins and HP-CD cyclodextrins were selected as carriers for inclusion complexation which were procured from Rajesh chemicals, Mumbai.

Sodium hydroxide pellets, concentrated HCl, potassium dihydrogen phosphate, acetone were procured from Rajesh chemicals, Mumbai.

Microorganisms used for antimicrobial activity

a. *Staphylococcus aureus*- NCIM 2079
b. *Escherichia coli*- NCIM 2109
c. *Pseudomonas aeruginosa*- NCIM 2036

Samples of microorganisms were procured from National Chemical Laboratory (NCL), Pune.

Instruments

a) FTIR-FTIR IR Affinity-1 Toshvin Analytical, Mumbai.
b) UV-UV-1700 Pharmaspec, Shimadzu, Japan.
c) DISSOLUTION-Electrolab TDT-08L Dissolution Tester
d) ANTIBIOTIC ZONE READER-Dolphin instruments, Mumbai
e) Shimadzu TGA-50 DSC instrument (Shimadzu Corporation, Japan)

Preparation of TMP-CDs inclusion complexes

The inclusion complexes of trimethoprim with β-cyclodextrins and HP-CD cyclodextrins were prepared in 1:1 and 1:2 ratio. The inclusion complexes of TMP were prepared by solvent evaporation, spray drying, kneading, and physical mixture methods [5].

Determination of drug content

UV spectrophotometry was used to determine the drug content of the binary system. The cyclodextrins: drug molar ratio was 1:1 and 1:2 in all system. Sample of the binary system were dissolved in phosphate buffer (pH 7.2) diluted to obtain ~ 10 µg/mL. The cyclodextrin: drug ratios would therefore remain 1:1 and 1:2 in the final solutions to calculate the drug content. The

absorbance of solutions were determined. From the absorbance, total drug content in the batches was calculated [6].

Phase solubility study

The phase solubility technique permits the evaluation of the affinity between *β*-CD as well as HP-*β*-CD and TMP in water. Phase solubility studies were performed according to the method reported by Higuchi and Connors TMP, in amount that exceeded its solubility, was taken into vial to which were added 25 mL of distilled water containing various concentration of *β*-cyclodextrin (1-5 mmol) and hydroxypropyl *β*-cyclodextrin (1-5 mmol). These flasks were sealed and shaken at 25 °C as well as 37 °C for 24 hrs. Subsequently, the aliquots were withdrawn, using a syringe and samples were filtered immediately through a whatman filter paper and diluted. The solution was analyzed by UV spectrophotometer against blank prepared in the same concentration of *β*-cyclodextrin as well as hydroxypropyl *β*-cyclodextrin in water so as to cancel any absorbance that may be exhibited by the *β*-cyclodextrin or hydroxypropyl *β*-cyclodextrin. From the absorbance the thermodynamic parameters were calculated [7].

Saturation solubility study

Weighed amount of TMP (pure drug), Inclusion complexes equivalent to 50 mg of the drug were separately introduced into 25 mL stoppered conical flasks containing 10 mL of distilled water. The sealed flasks were agitated on a rotary shaker for 24 hours at 27 °C and equilibrated for 2 days. An aliquot was passed through 0.45 μm membrane filter and the filtrate was suitably diluted and analyzed on a UV spectrophotometer [8].

Interaction studies

FT-IR spectroscopy

The inclusion complexes were subjected to IR analysis by the following method. An approximately minimum quantity (about 1 mg) of sample was thoroughly blended with adequate quantity of IR grade KBr (about 5 mg) in a mortar. The mixture was then made into thin films on a sample plate using a hand operated compression lever. The samples were then analyzed in a double beam IR spectrometer using KBr film as negative control (blank) [11].

UV-Vis spectroscopy

Stock solutions of pure drug and the formulations were prepared. It was serially diluted to fall within the calibration curve range. The maximum absorbance spectra of the TMP and formulations was measured at 200-400 nm in a UV–Vis spectrophotometer.

In vitro dissolution study

TMP and its inclusion complexes are subjected to in vitro dissolution study using electro lab TDT-08 L dissolution tester. TMP and its inclusion complexes were filled in capsules and dissolution study was carried out using basket method. The study was conducted at 25 rpm for 1 hour and sampling time interval was 5 min, 10 min, 20 min, 30 min, 40 min, 50 min and 60 min. 1 mL aliquots were withdrawn and volumes was made up to 10 mL with blank media and absorbance were determined by using UV-1700 pharmaspec, Shimadzu [12].

Screening for antimicrobial activity

Microorganism	Strain name	Strain reference
Gram positive bacteria	*Staphylococcus aureus*	NCIM 2079
Gram negative bacteria	*Escherichia coli*	NCIM 2109
	Pseudomonas aeruginosa	NCIM 2036
NCIM :National Chemical Laboratory (NCL), Pune 411008 [India]		

Agar disk-diffusion method

Agar disk-diffusion testing developed in 1940, is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. Nowadays, many accepted and approved standards are published by the clinical and laboratory standards institute (CLSI) for bacteria and yeast testing.

In this well-known procedure, agar plates were inoculated with a standardized inoculum of the test microorganisms. Then, filter paper discs (about 6 m min diameter), containing the test compound at a desired concentration were placed on the agar surface. The petri dishes were incubated under suitable conditions. Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones were measured [18-21].

A. **Culture used:** *B. subtilis*, *Staphylococcus aureus* and *Escherichia coli*.

B. **B. method used:** agar diffusion assay (disk diffusion method, disk size 6 mm).

C. **Media used:** microbiological media used for bacteria (*B. subtilis*, *Staphylococcus aureus* and *Escherichia coli*) is nutrient agar (Himedia).

Composition (gL-1): agar, 20; sodium chloride, 5.0; beef extract 10.0; peptone 10.0 (pH 7.2).

D. **Instruments used:** antib.

E. **biotic zone reader instrument:** (make dolphin).

Result and Discussion

Phase solubility study

Table 1. Phase solubility data of TMP with *β*-cyclodextrin and HP *β*-cyclodextrin

Drug	Carrier	Temp	Slope	Intercept	Ka	ΔG	ΔH	ΔS
TMP	*β*-cyclodextrin	25	-0.18876	0.11598	-0.2243	-2.0593	-2.06149	-0.6789
		37	-0.14039	0.1329	0.1231	-2.1905	-2.1905	-2.18
	HP *β*-cyclodextrin	25	-0.1314	0.13274	-0.8749	-1.0744	0.1074	0.00038
		37	-0.13799	0.11861	-1.0292	-0.8851	0.885102	0.000594

The results indicated that the solubility of TMP was found to increase with the concentration of the carrier added at 37 °C and decline with the concentration at 25 °C in the solubility was observed.

Assay data of inclusion complexes

From the assay data, it was clearly evident that the assayed drug content in the formulated inclusion complexes was found to be within the range of ± 10% of the theoretical amount. Assay data also indicates the methods used for formulation was suitable and reproducible in nature.

Table 2. Assay data of TMP inclusion complexes

Formulation method	Batches	Drug carrier ratio	Theoretical drug content		Assayed drug content	
			Amount [mg]	Expressed in %	Amount [mg]	Expressed in %
Kneading method	TMP: *β*-CD	1:1	25	100	26.01	104.04
	TMP: *β*-CD	1:2	16.66	100	15.95	95.73
	TMP: HP-*β*-CD.	1:1	25	100	24.41	97.64
	TMP : HP-*β*-CD	1:2	16.66	100	15.64	93.91
Co-evaporatin method	TMP: *β*-CD	1:1	25	100	22.94	91.78
	TMP: *β*-CD	1:2	16.66	100	15.01	90.13
	TMP: HP-*β*-CD.	1:1	25	100	22.46	89.85
	TMP: HP-*β*-CD	1:2	16.66	100	14.77	88.70
Spray drying	TMP: *β*-CD	1:1	25	100	25.81	103.24
	TMP: *β*-CD	1:2	16.66	100	16.89	101.38
	TMP: HP-*β*-CD.	1:1	25	100	24.25	97.00
	TMP: HP-*β*-CD	1:2	16.66	100	16.61	99.69
Physical mixture method	TMP: *β*-CD	1:1	25	100	24.34	97.38
	TMP: *β*-CD	1:2	16.66	100	16.34	98.07
	TMP: HP-*β*-CD.	1:1	25	100	23.63	94.53
	TMP: HP-*β*-CD	1:2	16.66	100	15.17	91.09

Saturation solubility studies

Saturation solubility was found increased with all selected formulations as compared to pure drug. Thus, saturation solubility studies proved the solubilization effect of the carrier on the solubility of the TMP.

Table 3. Saturation solubility of TMP inclusion complexes

Sr. No	Formulations	Amount Per ml [mg]
1	TMP	0.126
2	*β*-CD CEP 2	0.222
3	HP-CD CEP 2	0.232
4	*β*-CD KDG 1	0.257
5	HP-CD KDG 1	0.242
6	*β*-CD SD 1	0.277
7	HP-CD SD 1	0.231
8	*β*-CD PM 1	0.200
9	HP-CD PM 1	0.213

Interaction studies

FT-IR spectroscopy

Pure drug TMP and TMP-IC with *β*-cyclodextrin and hydroxypropyl cyclodextrin were subjected to FT-IR analysis. FT-IR was performed to investigate any possible interaction between TMP and *β*-cyclodextrin and hydroxypropyl cyclodextrin TMP has characteristic bands at 1634.3 and 1594.7 cm^{-1} which account for deformation of NH_2 group and stretching of aromation ring respectively. Deformation of C_9H_2 group appeared at 1458.7 cm^{-1}.

The characteristic peaks of model drugs were also present in sample spectra ruling out any possible interaction between the drug and carrier utilized in the formulations. This indicates that there is no interaction between TMP and cyclodextrins.

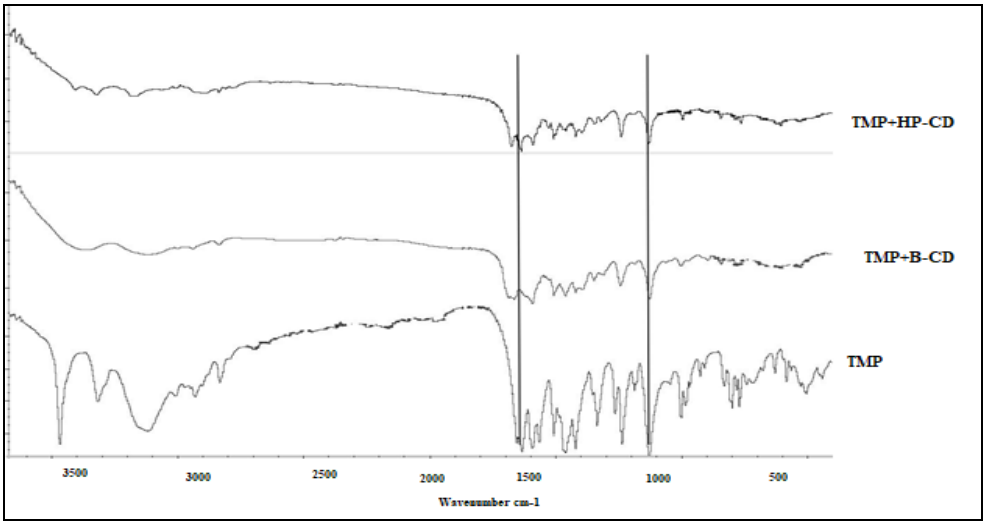


Figure 1. FT-IR spectra of TMP and TMP-IC with *β*-Cyclodextrin and hydroxypropyl cyclodextrin

UV analysis

The maximum absorbance spectra of the TMP and its inclusion complexes were measured at 200-400 nm in a UV–Vis spectrophotometer. The λ-max of pure drug and formulations was found to be nearly same i.e., 271 nm as shown in Figure 2. This indicates that there is no interaction between drug and cyclodextrins.

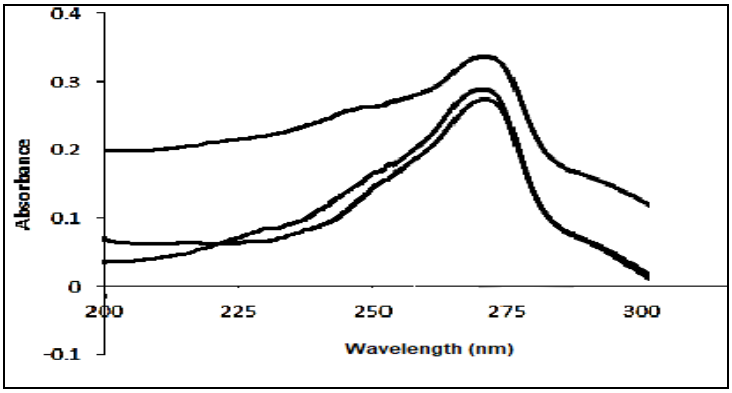


Figure 2. The λ-Max of TMP and TMP-IC

XRD analysis

XRD pattern of the pure TMP showed numerous sharp, narrow intense peaks, claiming its high crystallinity. On comparison of selected sample patterns with that of pure drug, it was observed that the intensity of peaks were found to be less in samples, and relative intensity percentage values were also found to be well correlating with interpretation guidelines.

The base of peaks in the sample were broader in nature as compared to pure drug. Relative intensity percentage values were also found to be less in samples confirming the reduction in crystallinity pattern can be treated as confirmation tool for reduction in crystallinity and phase transition (from crystalline to amorphous form) had occurred in the samples.

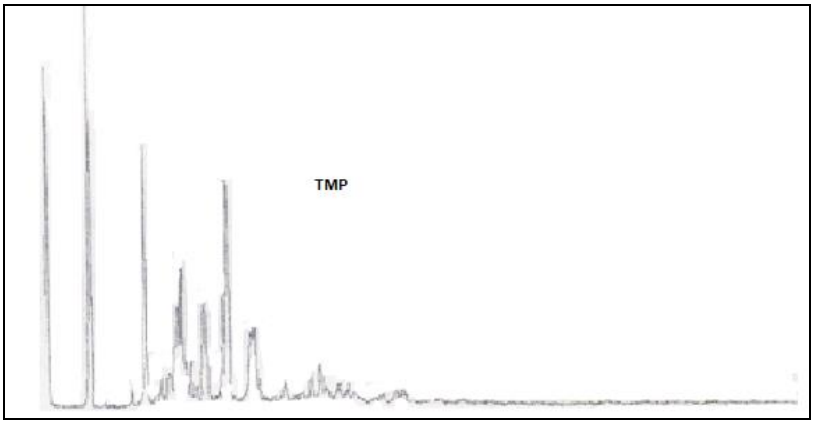


Figure 3. XRD analysis of TMP

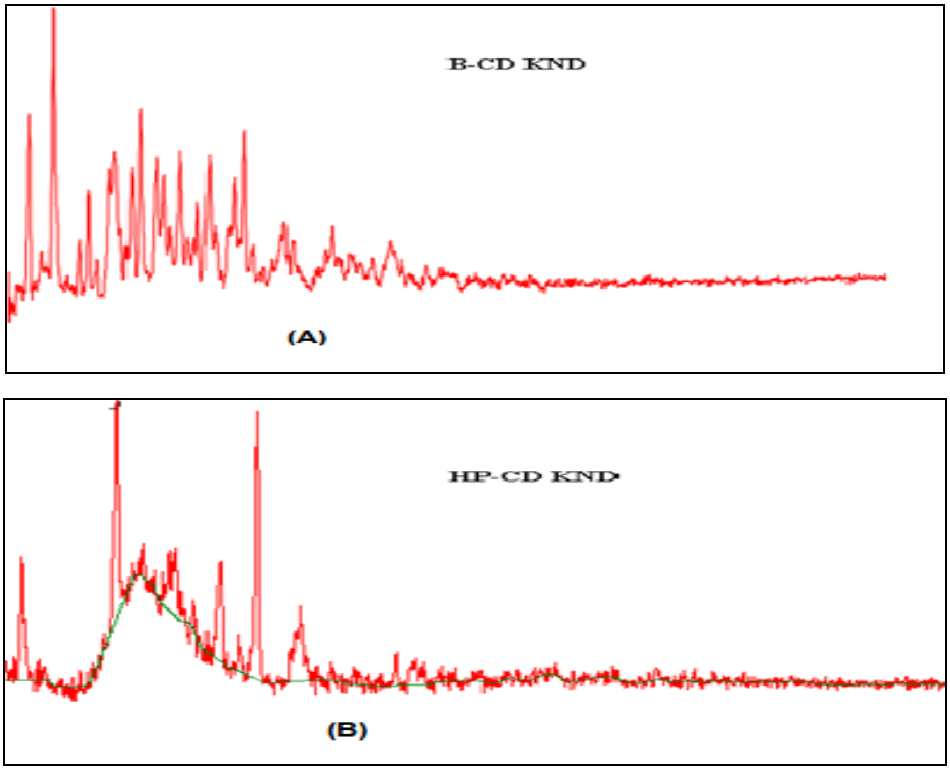


Figure 4. XRD analysis of TMP-IC prepared by kneading method. Sample (A)-TMP+B-CD. Sample (B)-TMP+HP-CD

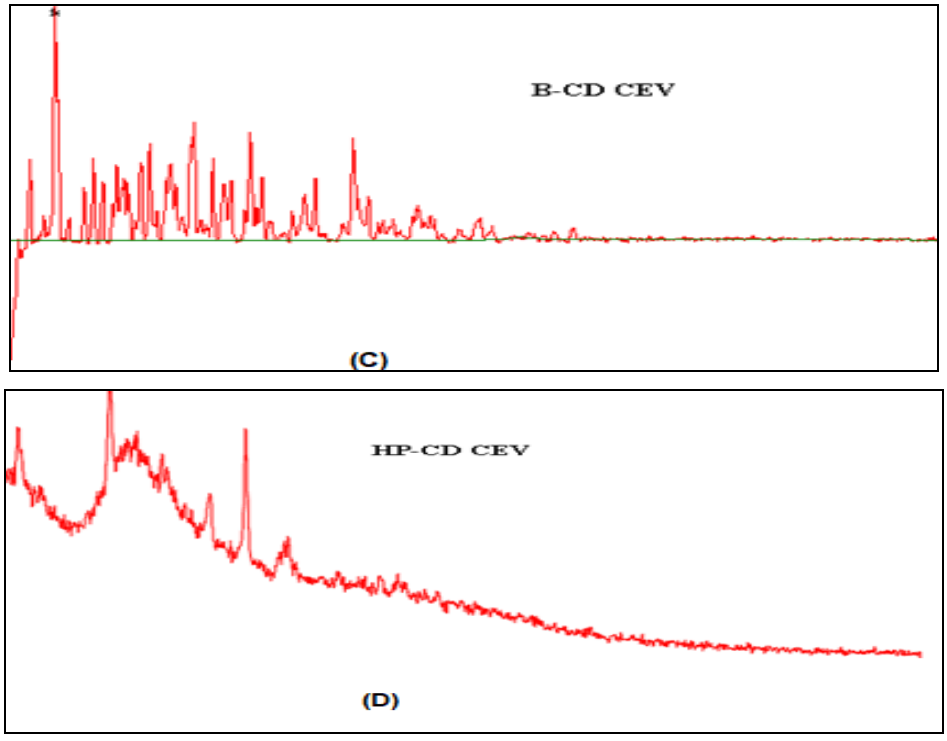


Figure 5. XRD analysis of TMP-IC prepared by co-evaporation method. Sample (C)-TMP+B-CD. Sample (D)-TMP+HP-CD

In vitro release study

Drug release data and release profiles of inclusion complexes of TMP with *β*-cyclodextrin and hydroxypropyl cyclodextrin are shown in Figure 6, 7, 8 and 9. A close observation of the data indicated that the release rate of all inclusion complexes was found to be higher than pure drug.

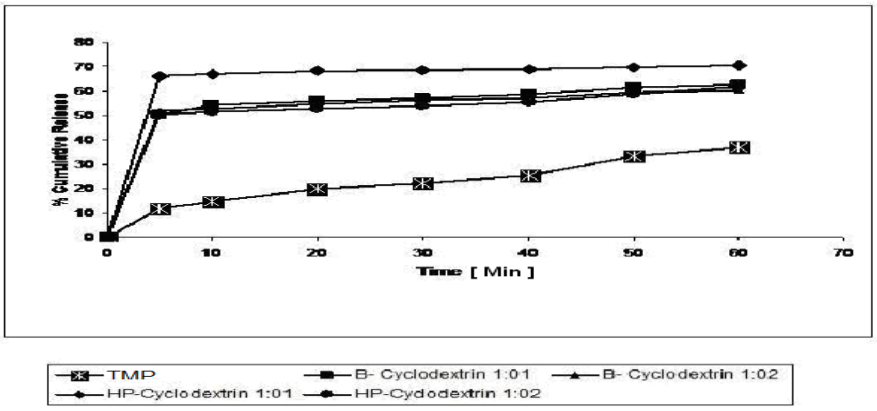


Figure 6. *In-vitro* release profile of IC prepared by kneading method compared with pure drug

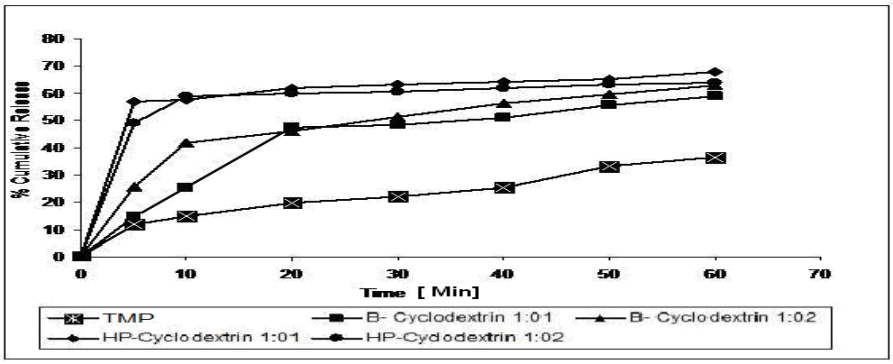


Figure 7. *In-vitro* release profile of IC prepared by co-evaporation method compared with pure drug

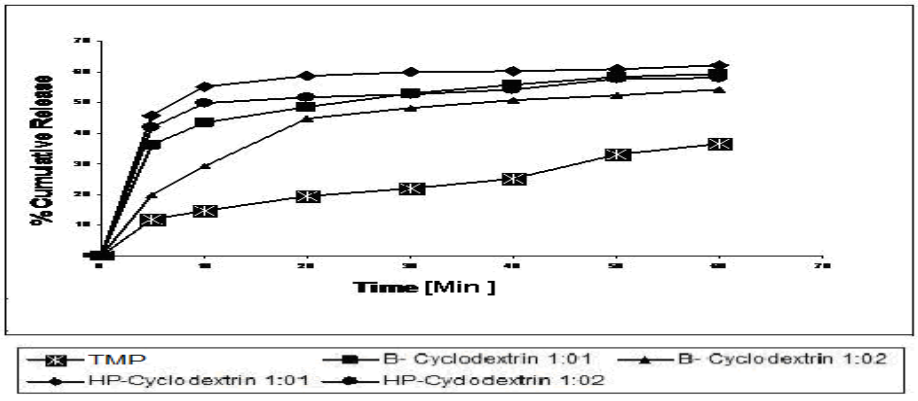


Figure 8. *In-vitro* release profile of IC prepared by spray drying method compared with pure drug

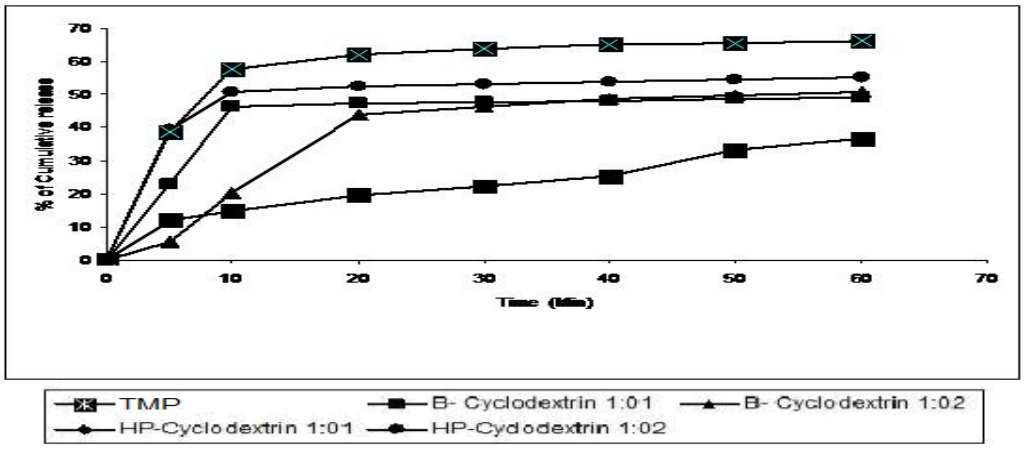


Figure 9. *In-vitro* release profile of IC prepared by physical mixture method compared with pure drug

Effect of method on TMP release

A close observation of release data revealed that the method utilized for formulation have a significant effect on the formation of IC. The three methods kneading, spray drying and physical mixture showed that IC formed at 1;1 ratio; whereas co-evaporation method formed a IC at 1:2 ratio indicating incorporation of more amount of carrier in the complex by co-evaporation method.

Effect of concentration of carrier

A close observation of release data revealed that the concentration of carrier have a significant effect on TMP release. The three methods kneading, spray drying and physical mixture showed that there is more drug release at 1:1 ratio. An increase in concentration tends to decrease the release rate than 1:1; whereas co-evaporation method showed more drug release at 1:2 ratio indicating incorporation of more amount of carrier in the complex**.**

DSC analysis

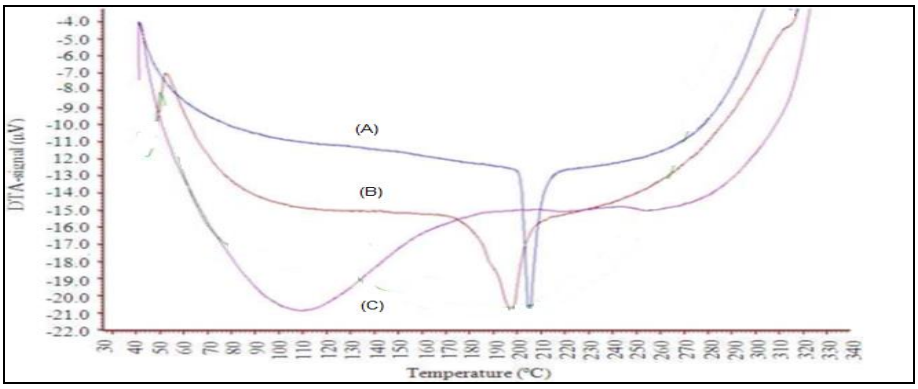


Figure 10. DSC thermogram of (A) TMP, (B) TMP+B-CD 1, (C) TMP+BCD 2

Pure drug TMP shows the sharp melting point endotherm for at approximately 200 °C. TMP inclusion complexes exhibited the complete loss of the sharp TMP melting point endotherms and a broadening of the respective CD melting point endotherm. The loss of the TMP melting point endotherm is suggested to be due to the encapsulation of the TMP into the internal cavity of the CD.

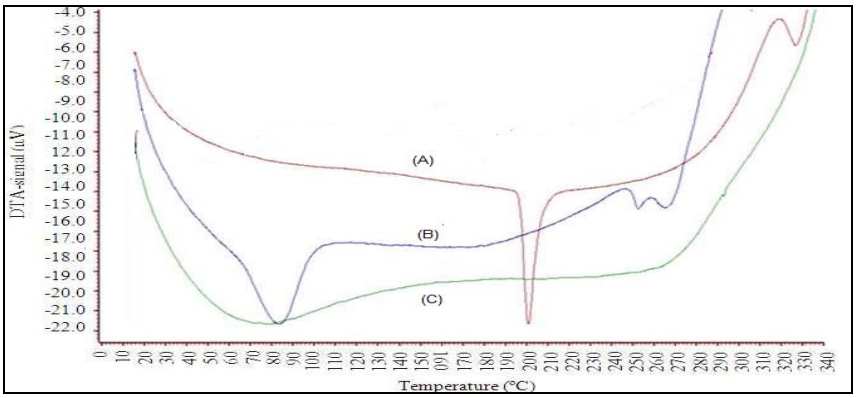


Figure 11. DSC thermogram of (A) TMP, (B) TMP+HPCD 1, (C) TMP+HPCD 2

Antimicrobial activity of TMP inclusion complexes

Antimicrobial activity of TMP and its inclusion complexes was carried out by using agar disk-diffusion method. The antimicrobial activity of TMP was found to be increased with *β*-CD and HPCD in 1:1 and 1:2 ratio against *E.coli, P. aeruginosa, S. aureus.* An increase in concentration of CDs increased the antimicrobial activity of TMP.

Table 4. Antimicrobial activity indicating zone of inhibition of TMP inclusion complexes

Sr. No.	Sample	*S. aureus*	*E. Coli*	*P. aeruginosa*
1	TMP	6.12 mm	5.96 mm	6.19 mm
2	TMP +*β*-CD 1:1	7.08mm	7.30 mm	6.50 mm
3	TMP+ *β*-CD 1:2	7.38 mm	7.34 mm	6.59 mm
4	TMP +HP-CD 1:1	12.51 mm	9.49 mm	8.63 mm
5	TMP +HP-CD 1:2	19.07 mm	16.24 mm	12.57 mm

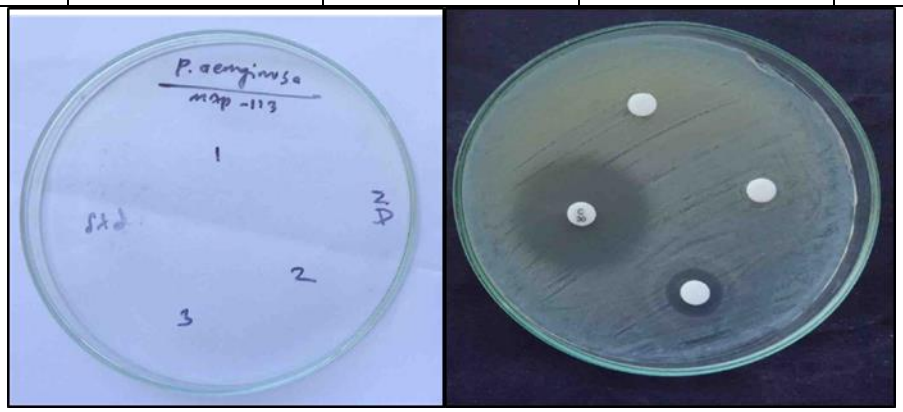


Figure 12. Antimicrobial activity of TMP-IC with *β*-CD and HP-CD against *P. aeruginosa*

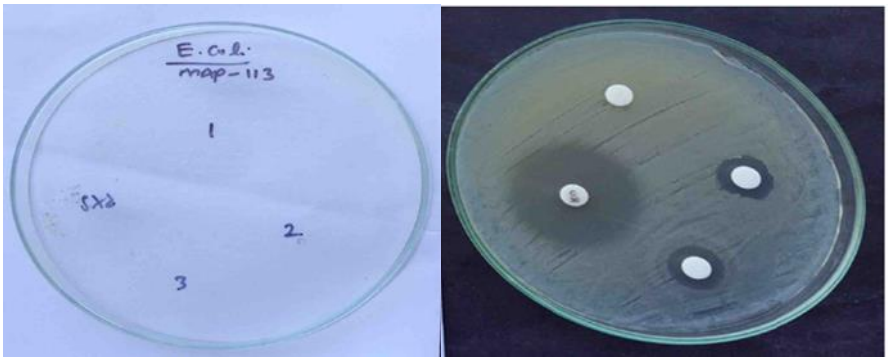


Figure 13. Antimicrobial activity of TMP-IC with *β*-CD and HP-CD against *E.*col*i*

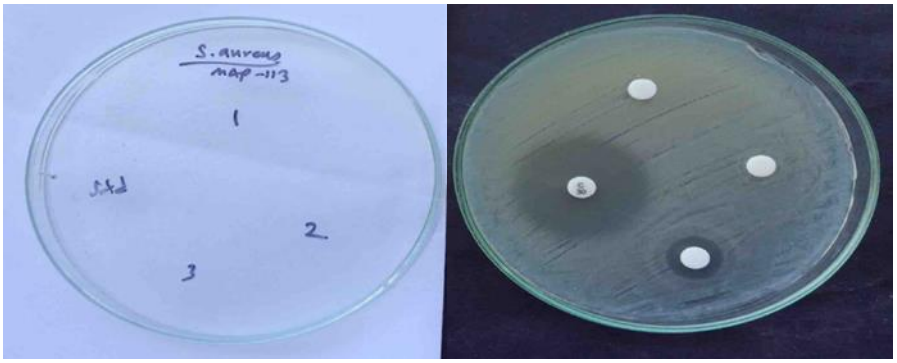


Figure 14. Antimicrobial activity of TMP-IC with *β*-CD and HP-CD against *S.aureus*

Conclusion

The prime objectives of preparation of inclusion complex by co-evaporation, kneading, spray drying and physical mixture method using *β*-cyclodextrin and hydroxypropyl-*β*-cyclodextrin were:

1) Enhancement of solubility of TMP by CD-inclusion complexation.
2) To study effect of CD on antimicrobial activity of TMP.
3) To study effect of concentration of CD on dissolution and antimicrobial activity of TMP.

1) Enhancement of solubility of TMP by CD-inclusion complexation

Phase solubility study results indicated that the solubility of TMP was found to increase with the concentration of the carrier added at 37 °C and decline with the concentration at 25 °C in the solubility was observed. The increase in dissolution behavior of TMP was observed with 1 hr and also kneading method was found to be best method among all methods.

XRD analysis findings confirmed the reduction in crystallinity in samples than pure drug. UV and I.R. analysis revealed that there was no interaction between the carriers and the drug used in formulation.

Thus solubility and dissolution of TMP significantly improved by forming its inclusion complexes with *β*-cyclodextrins and hydroxypropyl-*β*-cyclodextrins.

2) Effect of CDs on antimicrobial activity of TMP

The antimicrobial activity of TMP was found to be increased with *β*-CD and HPCD in 1:1 and 1:2 ratio against *E.coli, P.aeruginosa, S.aureus*. The antimicrobial activity of TMP was found to be more with HP-CD as compared to *β*-CD.

3) Effect of concentration of CD on dissolution and antimicrobial activity of TMP

The concentrations of carrier have a significant effect on TMP release. The three methods kneading, spray drying and physical mixture showed that there is more drug release at 1:1 ratio. An increase in concentration tends to decrease the release rate than 1:1; whereas co-evaporation method showed more drug release at 1:2 ratio indicating incorporation of more amount of carrier in the complex**.**

Antimicrobial activity of TMP and its inclusion complexes was carried out by using agar disk-diffusion method. The antimicrobial activity of TMP was found to be increased with *β*-CD and HP-CD in 1:1 and 1:2 ratio against *E.coli, P.aeruginosa, S.aureus.* An increase in concentration of CDs increased the antimicrobial activity of TMP.

Based on the findings from the present study we propose the following mechanisms which might be the reason for enhanced dissolution rate of model drug.

a. Reduced crystallinity in the samples i.e., phase transition from crystalline to amorphous.
b. Solublization effect of the carrier on the release of model drug.
c. Particle size reduction.

Thus, cyclodextrins offer a great potential as a drug carrier for TMP and may prove to be a valuable aid in improving the bio–availability and antimicrobial activity.

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