

Antibacterial Activity of β -Cyclodextrin and 2-Hydroxypropyl- β -Cyclodextrin Trimethoprim Complexes

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Abstract: Problem statement: Cyclodextrin complexation has previously been shown to improve the solubility and dissolution properties of trimethoprim; however, no report provides an account of the effect cyclodextrin complexation has on the antibacterial activity of this agent. **Approach:** β -cyclodextrin and 2-hydroxypropyl β -cyclodextrin inclusion complexes of trimethoprim were prepared and confirmed by differential scanning calorimetry and proton nuclear magnetic resonance. The *in-vitro* antibacterial activity, in terms of minimum inhibitory concentrations, of cyclodextrin-drug complexes were compared to uncomplexed free trimethoprim by a broth-microdilution method against several sensitive and resistant Gram-positive and Gram-negative bacteria. The effect of complexation on the apparent permeability coefficients was also determined using a Caco-2 permeability assay to account for potential alterations in bioavailability that could influence *in-vivo* antibacterial activity. **Results:** Inclusion complexation of trimethoprim with both unsubstituted and hydroxylated versions of β -cyclodextrin produced a reduction in the MIC₈₀ required to inhibit the growth of *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 4961, *S. epidermidis* ATCC 14990 and *E. coli* ATCC 25922 ($p > 0.05$). The effect was limited to bacteria normally susceptible to trimethoprim. Neither complex negatively affected the antibacterial activity of trimethoprim. Hydroxypropyl- β -cyclodextrin and β -cyclodextrin inclusion complexes significantly ($p < 0.01$) increased the apparent intestinal permeability of trimethoprim by 39.8 and 56.1%, respectively. Considering the effect cyclodextrin inclusion complexation has on the antibacterial activity of trimethoprim, the improved intestinal permeability of these complexes has the potential to improve the *in-vivo* antibacterial activity of the agent by enhancing the steady-state concentration of the drug when dosed orally. **Conclusion:** These results would suggest that physical complexation with either of these cyclodextrins would provide a feasible strategy to improve the pharmaceutical and pharmacokinetic properties of trimethoprim.

Key words: Antibacterial activity, trimethoprim, 2-hydroxypropyl, β -cyclodextrin, inclusion complex, antimicrobial activity, caco-2 cells, Differential Scanning Calorimetry (DSC), Minimum Inhibitory Concentrations (MIC), *S. aureus*

INTRODUCTION

Trimethoprim (TMP) [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] (Fig. 1) is a synthetic broad spectrum bacteriostatic agent, which inhibits bacterial dihydrofolate reductase. TMP, when used alone, remains clinically important for the treatment of uncomplicated urinary tract infections and mild-to-

moderate cases of acute or chronic prostatitis. When combined with other antimicrobials such as sulfamethoxazole, polymyxin B or dapsone the clinical usefulness of TMP extends to the prevention or treatment of *P. jiroveci*, *P. carinii*, *L. monocytogenes*, *Nocardia* spp, *Stenotrophomonas maltophilia*, *Burkholderia pseudomallei* (i.e., melioidosis), *Shigella* spp, *E. coli*, cerebral toxoplasmosis, pertussis and nocardia keratitis.

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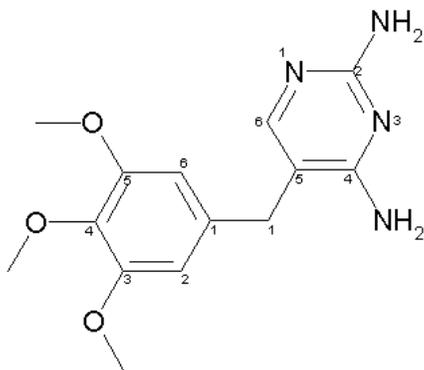


Fig. 1: Structure of trimethoprim

Previous research demonstrated that the aqueous solubility of TMP significantly increased (2.2-fold) when complexed with β -CycloDextrin (β CD) and the *in vitro* dissolution property of the β CD-TMP complex was superior to both TMP and the corresponding physical mixture of β -CD:TMP (Li *et al.*, 2005; Sousa *et al.*, 2005).

Cyclodextrin (CD) complexation has been shown to alter the activity of several β -lactam antibiotics against a diverse panel of clinical isolates (Athanasios *et al.*, 2003). The antibacterial activities of these CD-included antibiotics were shown to increase, particularly against Gram-negative bacteria. The nature and degree of substitution on CD macromolecules were proposed to be the predominant factors influencing the antimicrobial activity. (Athanasios *et al.*, 2003) Two mechanisms for these observed effects were suggested that may have relevance to our investigation. Firstly, several outer-membrane bacterial proteins similar to CymA may have facilitated the penetration of the CD-drug complexes (Pajatsch *et al.*, 1998; 1999; Orlik *et al.*, 2003). CymA is an identified component of the outer membrane of *Klebsiella oxytoca* that functions as a porin specific for CD (Orlik *et al.*, 2003). Secondly, CD molecules may have potentially destabilized the outer membrane of the bacteria, which eventually lead to an increase in the diffusion rate of the β -lactam antibiotics (Athanasios *et al.*, 2003).

Based on these findings, it is possible that CD complexation, in addition to altering the physicochemical properties of TMP, may alter its antimicrobial activity. Previous investigations did not provide an account of the biological properties of CD-TMP complexes. Given this, β CD and 2-hydroxypropyl β -CD (HP β CD) complexes of TMP were prepared and

the antibacterial activities of these complexes compared to uncomplexed TMP against a range of sensitive and inherently resistant bacteria. This investigation also compares the intestinal permeability of uncomplexed and complexed TMP to highlight potential pharmacokinetic differences that could influence *in vivo* antibacterial activity. Assessing the physicochemical attributes of complexes was not an objective of this investigation.

MATERIALS AND METHODS

Materials: TMP, β CD and HP β CD were purchased from Sigma-Aldrich (St Louis, USA). HPLC grade organic solvents were sourced from Merck (Darmstadt, Germany). All other chemicals were reagent grade. The water used throughout the investigation was deionised and filtered by a MilliQ-Academic system.

Preparation of the inclusion complexes of trimethoprim with CDs: Complexes were prepared by adding 1:1 Molar ratios of TMP and respective CD into 1 L litre of water and stirred until all solid materials dissolved. The resultant solutions were 0.45 μ m filtered (Millipore, Type HV, pore size 0.45 μ m) and transferred to round bottom flasks, frozen at -80°C (Sanyo) and subjected to freeze-drying using a Christ Alpha 1-4 LD freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany), at -57°C and 0.035 mbar. The freeze dried product was ground by mortar and pestle and sieved to obtain a relatively uniform particle size.

Characterisation of the inclusion complexes: Inclusion complex formation was confirmed by Differential Scanning Calorimetry (DSC), Infrared (IR) spectroscopy (data not shown) and proton nuclear magnetic resonance (^1H NMR) spectroscopy adapting previously reported protocols (Echezarreta-Lopez *et al.*, 2002; Sousa *et al.*, 2005) using methodology similar to that described by (Win *et al.*, 2010).

DSC measurements were performed using a Linseis STA Platinum Series analyser (Linseis Messgeraete GmbH, Germany). Pure TMP, β CD, HP β CD, respective equimolar physical mixtures and complexes were dried prior to thermal analysis. Samples (10 mg) were placed into an aluminium crucible fitted with a perforated lid and scanned over a temperature range of 30-400 $^{\circ}\text{C}$ at a heating rate of 5 $^{\circ}\text{C}$ min^{-1} .

^1H NMR spectra of TMP, β CD, HP β CD, respective equimolar physical mixtures and complexes

were acquired on a 600MHz Bruker Avance spectrometer equipped with a cryoprobe at 298K, in D₂O using DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid) as an internal standard.

Content analysis of trimethoprim-CD complexes: In order to compare antibacterial activities, it was necessary to determine the TMP content of formed complexes. A Shimadzu Biospec-mini spectrophotometer was used to determine the UV-absorbance profiles (230-400 nm) of TMP and equimolar solutions of CD-TMP complexes. TMP standards, with or without CDs, ranging from 5-75 µg/m L⁻¹ were prepared in acetonitrile and read at 292 nm. 10mg of each respective complex was dissolved in 100 mL of acetonitrile, transferred to quartz cuvettes and the absorbances read at 292 nm. The TMP concentration was extrapolated from the standard curve and respective equivalencies calculated.

Stock solution preparation for antibacterial assay: 5.12 mg mL⁻¹ stock solutions of TMP, βCD-TMP and HPβCD-TMP were prepared on the day of use by dissolving the calculated equivalent mass of TMP in 0.1 mL of 0.05 M hydrochloric acid and diluted with 0.9 mL⁻¹ of reverse osmosis water. All stock solutions were sonicated in a 45°C water bath for 10 min to aid in the dissolution of the powders. Stock solutions were serially diluted with broth to generate the required concentrations of 256, 128, 64, 32, 16, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 µg mL⁻¹.

Microorganisms: For the comparative study of antimicrobial activity a range of susceptible and inherently resistant Gram-positive and Gram-negative bacteria were tested: *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* NCTC 6571, methicillin resistant *Staphylococcus aureus* 49476, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus lugdunensis* ATCC 700328, *Streptococcus pneumoniae* ATCC 49619, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Neisseria meningitidis* ATCC 13077 and *Haemophilus influenzae* ATCC 49247.

Inoculum preparation: All cultures had been previously stored at -70°C and were subcultured overnight twice before use in each round of testing. Colonies from these subcultures were suspended in 0.9% sterile saline to create a turbidity equivalent to the 0.5 McFarland standard, measured in Vitek 2

suspension tubes in a Vitek 2 'Densichek' photometric turbidity meter (Biomérieux Inc., Durham, USA).

Antibacterial assay: A series of broth microdilution tests were performed to determine the Minimum Inhibitory Concentrations (MIC) of TMP, βCD and HPβCD complexes for each bacterial strain. Testing was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines for broth microdilution testing. About 100 µL of the antibiotic-containing media was pipetted into the appropriate 'U' shaped wells of a microtitre tray. The bacterial suspensions, prepared to a turbidity of 0.5 McFarland, were then diluted 1:10 with 0.9% saline and 5 µL of the diluted suspensions was added to each test well. Relevant positive (no drug) and negative controls (no bacteria) were included in each tray. Inoculated microtitre trays were covered to prevent contamination and evaporation of liquid media and were incubated at the appropriate atmosphere and temperature relevant to the organism being tested. Microtitre trays were removed from standardised conditions after an appropriate incubation period and read using a parabolic mirror stand to visually gauge the turbidity of each well compared to that of the negative and positive control wells. The MIC of each antibiotic dilution series in this study was read using the first dilution at which at least 80% of growth was inhibited (MIC₈₀). The experiment was repeated on three separate days. Quality control for all media, reagents, bacterial strains and equipment was performed, checked and found to be within acceptable limits as specified by the CLSI (where available) and as per the protocol of the National Association of Testing Authorities (NATA) accredited microbiology laboratory at the Princess Alexandra Hospital (Brisbane, Australia).

TMP High Performance Liquid Chromatography (HPLC) assay: The quantitative analysis of TMP was performed using an automated HPLC system, which consisted of a Varian Prostar 240 Quaternary Solvent Delivery Module, Varian Prostar 335 Photodiode Array Detector, Varian Prostar 430 Autosampler, equipped with a Varian Pursuit C18 reverse-phase column (250×4.6 mm, 5 µm particle size). The HPLC conditions used were adapted from methods previously reported (Gallego and Arroyo, 2002). The column was thermostated at 30°C and eluted TMP detected at wavelengths of 205 and 250 nm, respectively. The mobile phase consisted of 80:20 mixture of 10 mM aqueous phosphate buffer (pH 3) and acetonitrile. The flow rate was set at 1 mL min⁻¹ and 20 µL injection volume. Standard curves, used to extrapolate the

concentration of samples collected during the *in vitro* permeability assay, were generated for TMP ($R_t = 5.98 \pm 0.02$ min, $y = 140188x + 4622.9$, $R^2 = 0.9998$) and was linear from 0.25 μM to 10 μM . The limit of detection (LOD, signal-to-noise ratio 3-to-1) was 0.1 μM and the limit of quantification (LOQ, signal-to-noise ratio 10-to-1) was 0.25 μM .

***In vitro* permeability assay:** Caco-2 cells with a passage number 38 were grown on polycarbonate inserts for 29 days to form cell monolayers with tight junctions. The Transepithelial Electrical Resistance (TEER) across inserts was measured to determine the integrity of cell monolayers. Prior to the experiment, the cell monolayers were rinsed with the Hank's Balanced Salt Solution (HBSS) with a pH of 7.2. All the TEER measurements were above 250 Wcm^2 , indicating intact cell monolayers suitable for investigating intestinal permeability. The permeability experiments were performed in the apical to basolateral direction. Briefly, at the start of the experiment, 200 μM solutions of TMP, $\beta\text{CD-TM}$ and $\text{HP}\beta\text{CD-TMP}$ prepared in HBSS were added to the apical side of the cell monolayers. At 30 min intervals over a period of 3 hours, the inserts were transferred to new wells filled with fresh HBSS. Samples, taken from the basolateral side of the cell insert, were analysed by HPLC to determine the TMP concentration. The method was performed in triplicate.

Data analysis: The median MIC_{80} was calculated for each bacterial species. The Kruskal-Wallis non-parametric analysis of variance on ranks was used to determine whether there were significant differences among susceptibilities to uncomplexed TMP to CD-TMP for each bacterial species. Mann-Whitney tests were performed to determine specific differences between TMP and each CD complex (Mann-Whitney test). Differences were considered significant when $p < 0.05$.

For the *in-vitro* intestinal permeability assay, extrapolated concentrations were used to determine the P_{app} for each compound which was calculated using the formula:

$$P_{app} = \frac{\Delta Q}{\Delta t} \frac{1}{AC_o}$$

Two-tailed t-test was used to assess the level statistical differences in intestinal permeabilities of uncomplexed trimethoprim to CD complexes. Differences were considered significant when $p < 0.05$.

RESULTS

Content analysis of TMP-CD: The UV wavelength scans (230-400 nm) provided evidence that CDs do not significantly affect the UV-absorption profile of TMP at any wavelength ($p < 0.05$; $n = 3$). No significant difference was observed for slope or linearity ($y = 19.246x$; $R^2 = 0.998$ for TMP), ($y = 19.627x$; $R^2 = 0.9973$ for $\text{HP}\beta\text{CD-TMP}$) and ($y = 19.119x$; $R^2 = 0.9967$ for $\beta\text{CD-TMP}$) at a wavelength of 292 nm ($p < 0.05$; $n = 3$). Spectrophotometric analysis at 292 nm confirmed that 10 mg of $\beta\text{CD-TMP}$ and $\text{HP}\beta\text{CD-TMP}$ complexes were equivalent to 1.97 ± 0.03 mg and 1.49 ± 0.05 mg of uncomplexed free TMP, respectively.

Characterization of the inclusion complexes: DSC provided evidence supporting the formation of CD-TMP inclusion complexes (Fig. 2 and 3). The sharp melting point endotherm for TMP was identified at approximately 200°C. The broad melting point endotherms for βCD and $\text{HP}\beta\text{CD}$ were identified at approximately 85 and 110°C, respectively. Evaluation of the respective physical mixes (1:1 Molar ratio) revealed individual endotherms for TMP, βCD and $\text{HP}\beta\text{CD}$. 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 endotherm is suggested to be due to the encapsulation of the drug into the internal cavity of the CD (i.e., inclusion complex) (Li *et al.*, 2005).

The results of the NMR analyses of TMP and respective inclusion complexes are shown in Table 1 referenced to DSS (0.0 ppm). NMR analysis provides further evidence regarding the inclusion and orientation of TMP within the host (βCD or $\text{HP}\beta\text{CD}$). ^1H NMR evidence for the formation of an inclusion complex is provided by changes in the chemical shift ($\Delta\delta$) of protons associated with either the CD or TMP, reflecting changes in chemical environment on encapsulation. The data clearly show that the inclusion of trimethoprim into either the βCD or $\text{HP}\beta\text{CD}$ results in a change in chemical shift (shielding effect) of the TMP resonances on complexation.

Table 1: ^1H NMR resonance chemical shifts δ (ppm) of C-H proton in TMP and $\Delta\delta$ (ppm) of C-H protons in TMP complexed with either βCD or $\text{HP}\beta\text{CD}$ in D_2O referenced to DSS

^1H	TMP δ_{ppm}	TMP/ $\Delta\delta$ $\beta\text{-OH-CD}$	TMP/ $\Delta\delta$ $\beta\text{-CD}$
6 $\hat{}$	7.677	-0.131	-0.164
1 $\#$	3.710	-0.094	n.d. ¹
2*, 6*	6.610	-0.112	-0.153
3*, 5*	3.818	-0.089	n.d.
4*	3.754	-0.093	n.d.

¹n.d. due to overlap with the $\beta\text{-cyclodextrin}$ signals; $\hat{}$ pyrimidine C-H proton; $\#$ alkyl C-H proton, *benzyl O-C-H

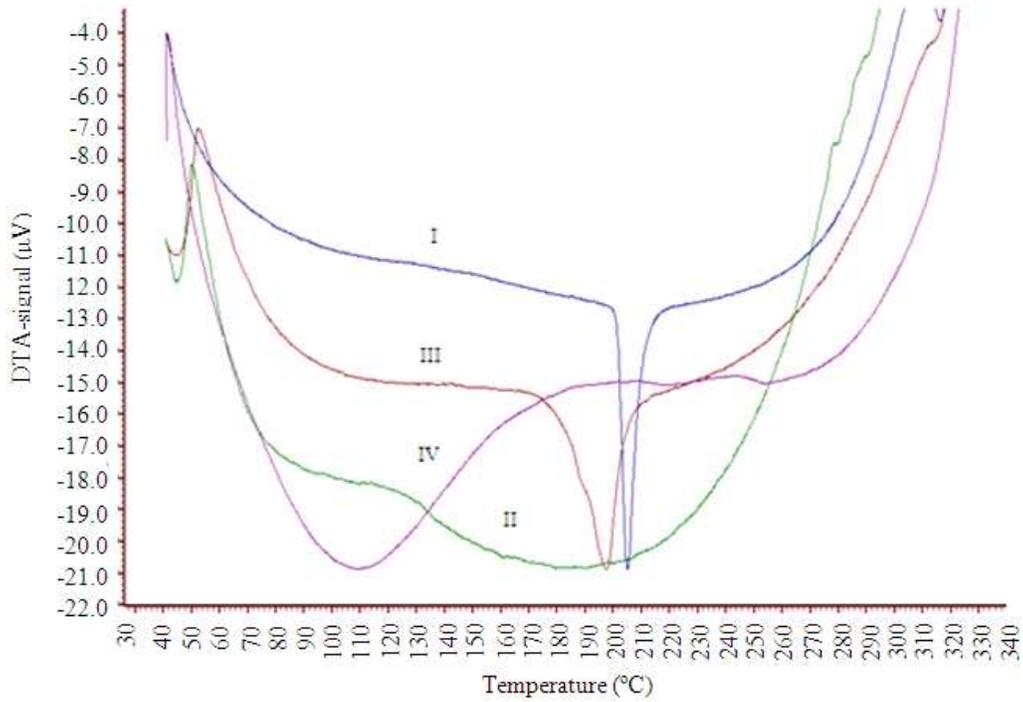


Fig. 2: Differential scanning calorimetry (DSC) thermogram for TMP (I); β CD (II); physical mix (III); β CD-TMP complex (IV)

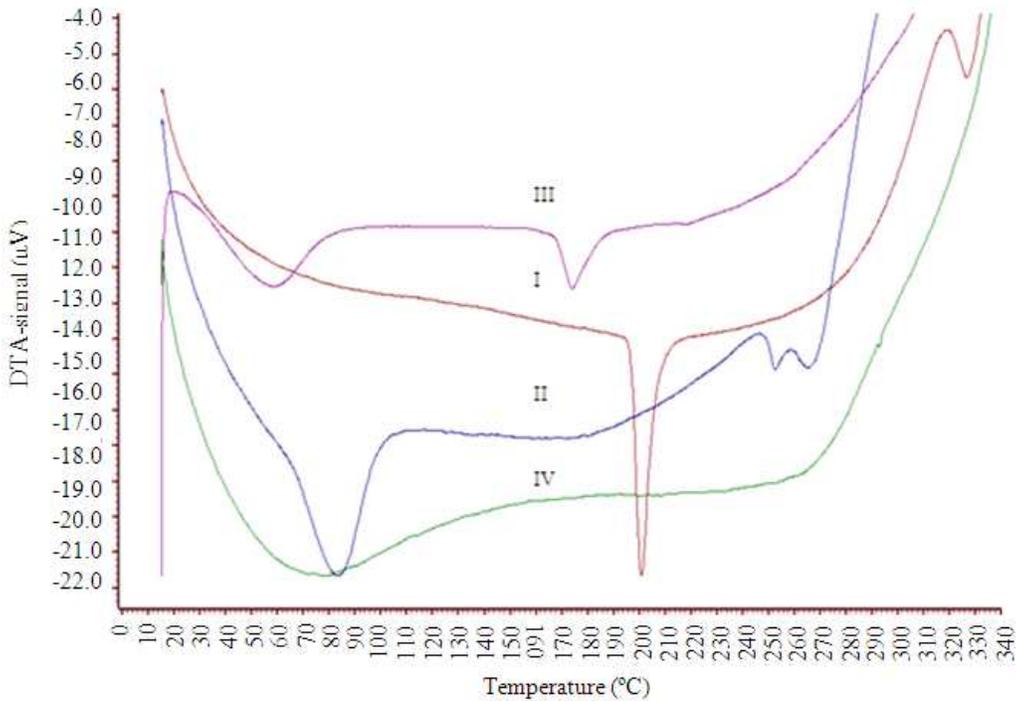


Fig. 3: Differential scanning calorimetry (DSC) thermogram of TMP (I); HP β CD (II); physical mix (III); HP β CD-TMP complex (IV)

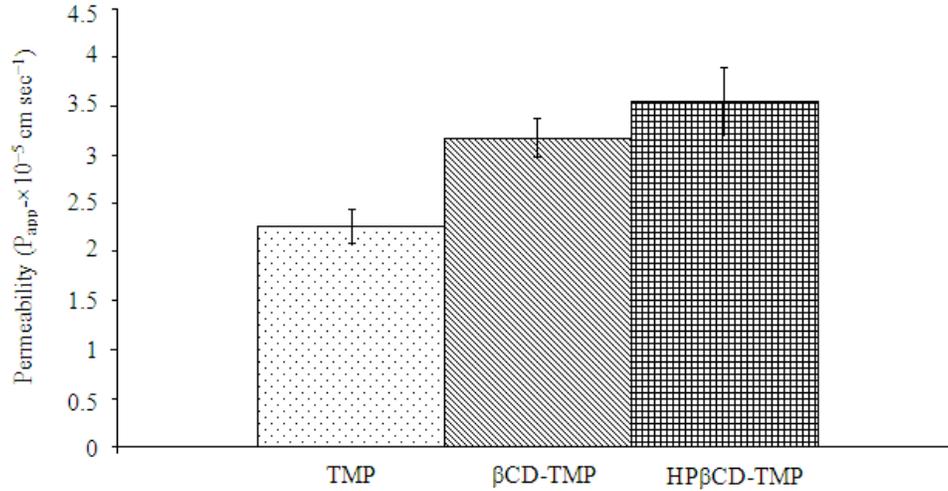


Fig. 4: Mean permeability of TMP (n = 4), β CD-TMP (n=3) and HP β CD-TMP (n = 3). **: p<0.01 for TMP Vs β CD-TMP; #: p<0.01TMP Vs HP β CD-TMP

Table 2: MIC₈₀ (mg mL⁻¹) of TMP and equivalent concentrations of β CD-TMP and HP β CD-TMP complexes against selected Gram-positive and Gram-negative bacteria

Organism (NCTC/ATCC Strain)	TMP median (range)	β CD-TMP median (range)	HP β CD-TMP median (range)
<i>Staphylococcus aureus</i> 29213	2 (1-2)	1 (-)	1 (0.5-1)
<i>Staphylococcus aureus</i> 6571	1 (0.5-1)	1 (0.5-1)	0.25 (0.25-1)
methicillin resistant <i>Staphylococcus aureus</i> 49476	1 (0.5-1)	1 (0.5-1)	0.5 (0.25-1)
<i>Staphylococcus epidermidis</i> 14990	0.125 (0.125-0.5)	0.125(0.125-0.25)	0.125 (0.125)
<i>Staphylococcus lugdunensis</i> 700328	8 (4-8)	8(4-8)	8 (4-8)
<i>Streptococcus pneumoniae</i> 49619	4 (2-4)	2(2-4)	2 (2-4)
<i>Escherichia coli</i> 25922	0.5 (0.5-1)	0.5 (-)	0.25 (0.125-0.5)
<i>Haemophilus influenzae</i> 49247	0.125 (-)	0.125 (-)	0.125 (-)
<i>Pseudomonas aeruginosa</i> 27853	>256 (-)	>256 (-)	>256 (-)
<i>Neisseria meningitidis</i> 13077	64 (32-64)	64 (32-64)	64 (32-64)

Antibacterial activity of CD-TMP complexes: The median (range) MIC₈₀ for TMP, β CD-TMP and HP β CD-TMP against Gram-positive and Gram-negative bacteria are presented in Table 2. CD complexation was shown to marginally increase the *in-vitro* antibacterial activity of TMP against bacteria normally susceptible to this agent. Inclusion complexation of TMP with both unsubstituted and hydroxylated versions of β CD appeared to reduce the median MIC₈₀ required to inhibit the growth of *S. aureus* 29213, *S. pneumoniae* 4961 and *E. coli* 25922 (p>0.05) (Table 2). This effect was also observed for HP β CD-TMP against *S. aureus* 6571 and methicillin resistant *S. aureus* 49476 (p>0.05) (Table 2). The median MIC₈₀ range for HP β CD-TMP against *S. epidermidis* 14990 decreased relative to TMP alone and the β CD-TMP complex (p>0.05). Neither CD-TMP inclusion complex affected the antibacterial activity against *H. influenzae* 49247. CD inclusion complexes of TMP also produced no alteration in the activity of

TMP against inherently resistant Gram-positive bacteria such as *S. lugdunensis* 700328 and Gram-negative bacteria such as *P. aeruginosa* 27853 and *N. meningitidis* 13077. With exception of *S. aureus* 49476, HP β CD inclusion complexes appeared to reduce the median MIC₈₀ of TMP against normally susceptible bacteria more than β CD, Table 2. The lack of statistical significance is likely due to insufficient statistical power of this study as a result of the small sample size (n = 3) and the degree of variance produced by CD complexation.

In-vitro intestinal permeability assay: Results are presented in Fig. 4. β CD and HP β CD inclusion complexation significantly increased the *in-vitro* apparent intestinal permeability of TMP by 39.8% and 56.1%, respectively (p<0.01; n = 3). Uncomplexed free TMP displayed lower apparent permeability (P_{app} 2.27 $\times 10^{-5}$) when compared to either CD-TMP complex (β CD-TMP P_{app} 3.17 $\times 10^{-5}$ and HP β CD-TMP P_{app} 3.59

$\times 10^{-5}$). The mean P_{app} of HP β CD-TMP, despite being higher, was not significantly different from β CD-TMP ($P > 0.05$; $n = 3$).

DISCUSSION

The formation of inclusion complexes between TMP and β CD or HP β CD were confirmed by DSC (Fig. 2 and 3) and $^1\text{H-NMR}$ (Table 1). CD inclusion complexation resulted in improvements in the antibacterial activity of TMP against normally sensitive bacteria that were not significant. These results suggest that it is unlikely that CD inclusion complexation would have any influence on the spectrum of organisms affected by TMP and as such would have little bearing on the clinical indication of this agent. From a formulatory perspective, these results highlight the fact that TMP, at minimum, retains its original activity against susceptible bacteria when complexed with these CDs.

The statistically significant improvement in the intestinal permeabilities of both β CD inclusion complexes relative to uncomplexed free TMP, however, may have marked implications for the *in-vivo* antibacterial activity. The 39.8 and 56.1% increase in apparent intestinal permeability observed for β CD-TMP and HP β CD-TMP (Fig. 4) relative to uncomplexed free TMP could result in significantly higher steady-state plasma concentrations of the drug when administered orally. Extrapolation of these *in-vitro* findings to *in-vivo* is supported by comprehensive validation studies previously performed (Artursson 1990). A reliable correlation between the oral absorption in humans and the apparent permeability coefficient, obtained from the Caco-2 permeability assay, for 20 structurally diverse chemicals, was demonstrated by the investigator (Artursson and Karlsson, 1991; Artursson *et al.*, 2001). Drugs with a P_{app} above 1×10^{-5} cm sec $^{-1}$ in Caco-2 cell studies are considered to be highly absorbed while drugs with P_{app} below 2×10^{-6} cm/s are considered to be poorly absorbed *in-vivo* (Rinaki *et al.*, 2003). Considering the effect CD inclusion complexation has on the antibacterial activity of TMP, the improved intestinal permeability of CD-TMP complexes has the potential to improve the *in-vivo* antibacterial activity of TMP by enhancing the steady-state concentration of the drug when dosed orally, reduce emergence of resistance, or at minimum reduce the oral dose required to attain the desired steady-state plasma concentration to inhibition the growth of bacterial for which TMP is indicated.

CONCLUSION

These results in combination with the improvements in physicochemical properties previously reported (Li *et al.*, 2005; Sousa *et al.*, 2005) would suggest that physical complexation with either of these CDs would provide a feasible alternative strategy to improve the pharmaceutical and pharmacokinetic properties of TMP. This would be particularly applicable to the formulation or compounding of solution-state dosage forms such as eye drops required for the treatment of ocular pathogens. The clinical relevance of these results is limited by the *in-vitro* nature of the tests performed and future investigations should provide comparisons in an *in-vivo* model to validate the conclusions drawn from this investigation.

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