DOI 10.5012/bkcs.2010.31.11.3460

Solubility Enhancement and Antibacterial Activity of Chloramphenicol Included in Modified β-Cyclodextrins

Antonio Zuorro, Marco Fidaleo,[†] and Roberto Lavecchia[*]

Department of Chemical Engineering, "La Sapienza" University, Via Eudossiana 18, 00184 Rome, Italy
[*]E-mail: roberto.lavecchia@uniroma1.it
[†]Department of Cellular and Developmental Biology, "La Sapienza" University, P. le Aldo Moro 5, 00185 Rome, Italy
Received June 7, 2010, Accepted September 15, 2010

Key Words: Chloramphenicol, Cyclodextrins, Antibacterial activity, Ocular pathogens, Eye drops

Chloramphenicol (CHL) is a broad-spectrum antibiotic exhibiting bacteriostatic activity against many Gram-negative and Gram-positive bacteria as well as chlamydiae, rickettsiae and mycoplasmas.[1] CHL is widely used for the treatment of superficial eye infections and, because of its low cost and recognized efficacy, it is one of the most popular antibiotics in developing countries. Its mechanism of action has been elucidated and found to rely on the inhibition of peptidyl transferase, an RNA enzyme that catalyzes peptide bond formation between adjacent amino acids during protein synthesis.[2] Although the CHL molecule is quite small (MW = 323.13 Da) and sufficiently stable under ordinary storage conditions, the presence of a nitrobenzene moiety (Figure 1) makes this compound scarcely soluble in water. As a result, hydrophilic prodrugs (e.g., CHL sodium succinate and CHL palmitate) or oily vehicles are generally used for the preparation of pharmaceutical compositions.[4]

In this study we have explored the possibility of including CHL in water-soluble cyclodextrins (CDs) and using the resulting complex to formulate aqueous eye drops at high antibiotic concentration. CDs are cyclic oligosaccharides consisting of six to eight glucopyranose units (α-, β- and γ-cyclodextrins, respectively) joined together by α-(1,4)-glycosidic bond to form a toroidal structure with a hydrophobic cavity and a hydrophilic outer surface.[5] We used two chemically modified β-cyclodextrins, 2-hydroxypropyl-β-cyclodextrin (HPβCD) and methyl-β-cyclodextrin (MβCD), whose main characteristics are illustrated in Table 1. They were selected because of their commercial availability as pharmaceutical-grade products and their excellent aqueous solubility as compared to that of the unsubstituted β-CDs. In addition, their use in ocular preparations has been proven to be safe, even at concentrations as high as 45% w/w.[6] In the ophthalmic field, the availability of a CHL-CD conjugate would offer several advantages over the existing formulations, the most important being the avoidance of use of ointments or oily vehicles[7] and the increased *in-vivo* delivery of CHL, due to the reported ability of CDs to cross the corneal epithelial barrier.[8] However, no study has so far investigated the effects of CD inclusion on the antimicrobial activity of CHL. This is a key point to be assessed, as a too weak binding would not ensure an adequate solubilization of CHL in aqueous media. On the other hand, a too strong interaction between the CD and the drug could modify its antibiotic activity and/or reduce the amount of drug released to the bacterial target sites.

As a first step towards the above mentioned objective, we determined the stoichiometry and the stability constants of the complexes. The effect of the presence of increasing amounts of HPβCD on the UV absorption spectrum of CHL is shown in Figure 2. Similar trends were seen with MβCD. The observed increase in the apparent CHL solubility on addition of CDs is indicative of the formation of a complex between CHL and the two CDs. For both of them, the phase-solubility diagram was of A_L type, i.e., the aqueous solubility of CHL increased linearly with the CD concentration, and the slope was < 1 (Figure 3), suggesting a stoichiometry of 1:1 for the complexation process.[9] Accordingly, the solubility isotherm for such process (CHL + CD ⇔ CHL·CD) can be written as:

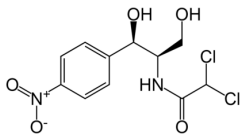

Figure 1. Molecular structure of chloramphenicol.

Table 1. Main characteristics of the modified β-cyclodextrins

Property	CAVASOL® W7-HP	CAVASOL® W7-M
Molecular weight	1400	1310
Substitution degree	0.59 - 0.73	1.70 - 1.90
Melting point	120 - 160 °C	164 - 172 °C
Solubility in water at 25 °C	2300 g L^{-1}	> 750 g L^{-1}

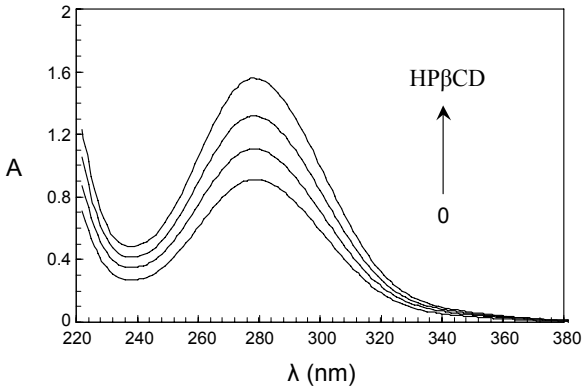

Figure 2. Changes in the UV absorption spectrum of 18 mM CHL in water at 25 °C upon addition of 5, 10 and 20 mM HPβCD.

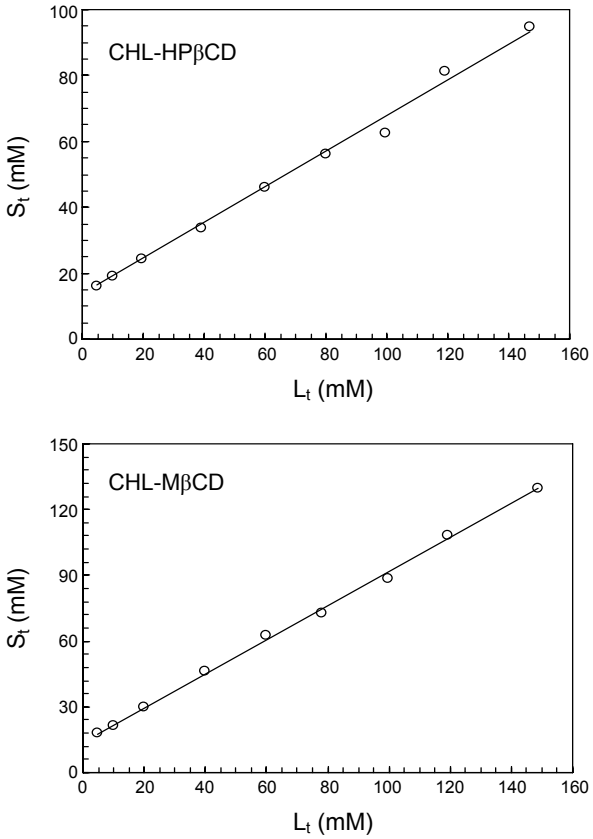

Figure 3. Phase-solubility diagram of CHL-HPβCD and CHL-MβCD in water at 25 °C. S_t and L_t are the total concentrations of substrate and ligand, respectively.

$$S_t = s_0 + \frac{K_{1:1}s_0L_t}{1 + K_{1:1}s_0} \quad (1)$$

where S_t and L_t are the total concentrations of substrate (CHL) and ligand (CD), respectively, s_0 is the substrate solubility in the liquid and $K_{1:1}$ is the equilibrium constant for complex formation. s_0 and $K_{1:1}$ were estimated by correlating the S_t *vs*. L_t data. The results are presented in Table 2. From the values of $K_{1:1}$ and ΔG^0 it can be seen that the CHL-MβCD complex is more stable than the CHL-HPβCD one ($K_{1:1}$ = 259.5 and 86.3 M^{-1}, respectively) which means that, for a given cyclodextrin concentration, MβCD allows more CHL to be solubilized. Overall, the apparent solubility of CHL was increased from about 4.4 g L^{-1}, in water, to up to 30.7 g L^{-1}, in the presence of HPβCD, or up to 42.0 g L^{-1} with MβCD.

The formation of a 1:1 complex is consistent with an inclusion-type interaction where the CD molecule fully envelops the nitrobenze moiety of CHL. In this regard, it may be interesting

Table 2. Estimated thermodynamic parameters for the inclusion of CHL in HPβCD and MβCD

Parameter	CHL-HPβCD	CHL-MβCD
s_0 (g L^{-1})	4.41 ± 0.37	4.41 ± 0.37
$K_{1:1}$ (M^{-1})	86.3 ± 4.8	259.5 ± 14.0
ΔG^0 (kJ mol^{-1})	−11.04 ± 0.14	−13.77 ± 0.13

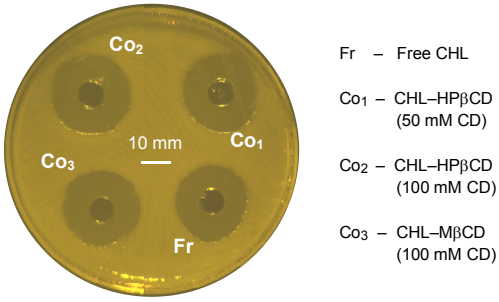

Figure 4. Inhibition zones against *P. mirabilis* produced by 30 μg of free or complexed CHL in sterile isotonic eye drops.

to consider the results of Chen *et al.*, who investigated the interactions between nitrobenzene and native β-CDs by ^{1}H NMR spectroscopy and X-ray crystallography.[10] The authors found that the stoichiometry of the complex was 1:1 and that the nitrobenzene molecule was completely included within the cyclodextrin cavity. At 25 °C and neutral pH, the equilibrium constant for complex formation was 153.8 M^{-1}, a value that is intermediate between those determined in this study for CHL inclusion in HPβCD (86.3 M^{-1}) and MβCD (259.5 M^{-1}). These differences are most likely due to the presence of a polar group, in the CHL molecule, attached to the nitrobenzene moiety (Figure 1) and to the occurrence of interactions between this group and the β-CD substituents.

To evaluate the antimicrobial properties of the complexed CHL, two Gram-negative bacteria (*Escherichia coli* and *Proteus mirabilis*) and two Gram-positives (*Staphylococcus aureus* and *Staphylococcus epidermidis*) were used. They are among the bacterial pathogens most commonly associated with ocular infections.[11] *S. epidermidis* was isolated at "Tor Vergata" University Hospital (Rome, Italy) from an infected wound and identified as a methicillin-resistant (MRSE) strain. It was included among the pathogens tested because it is a major cause of conjunctivitis, keratitis and endophthalmitis all over the world.[12] Eye drop formulations were prepared from solutions with varying amounts of CDs. In particular, the antibiotic eye drops examined (A-D) contained: 50 mM HPβCD and 40.7 mM CHL (A); 100 mM HPβCD and 67.8 mM CHL (B); 50 mM MβCD and 52.7 mM CHL (C); 100 mM MβCD and 91.7 mM CHL (D). Controls were also prepared consisting of free CHL, of single CDs and of the ophthalmic solution. Appropriate amounts of these solutions were transferred in the agar plates so as to provide 30 μg CHL in each well. An example of the results obtained is shown in Figure 4. No inhibition of bacterial growth was observed in negative controls, while eye drop formulations with the free or complexed drug showed significant activity against all the four pathogens. The resulting inhibition zone diameters ranged from 18.5 to 26 mm (Table 3). Interestingly, CHL showed activity in the complexed as well as uncomplexed form against MRSE, whose prevalence in hospitalized and community-based patients is increasing at a rapid rate.[13]

From these results, three main conclusions can be drawn. First, the antibiotic activity of CHL is not impaired upon CD complexation. Second, there does not appear to be any significant difference in the behavior of CHL-HPβCD and CHL-

Table 3. Diameters of pathogen inhibition zones (mm) for eye drops with free (CHL) or complexed (CHL-HPβCD, CHL-MβCD) chloramphenicol. The superscripts '*a*' and '*b*' denote drug complexes prepared from solutions containing 50 or 100 mM cyclodextrins, respectively

Pathogen	CHL	CHL-HPβCD	CHL-MβCD
E. coli	21.0 ± 0.0	25.0 ± 0.0^a 24.5 ± 0.7^b	24.0 ± 0.0^a 26.0 ± 0.0^b
P. mirabililis	24.5 ± 0.7	25.0 ± 0.0^a 25.5 ± 0.7^b	22.5 ± 0.7^a 25.0 ± 0.0^b
S. aureus	18.5 ± 0.0	24.5 ± 0.7^a 23.5 ± 0.7^b	21.5 ± 0.7^a 21.5 ± 0.7^b
MRSE	22.5 ± 0.7	24.5 ± 0.7^a 23.0 ± 0.0^b	22.0 ± 0.0^a 22.0 ± 0.0^b

MβCD complexes, suggesting that the chemical nature of substituents on the βCD ring has a limited effect on the activity of the complexed drug. Finally, eye drops prepared from solutions at different CD concentration (50 or 100 mM) displayed very similar antibacterial activity. It can therefore be inferred that the interactions between CHL and the two CDs are sufficiently strong to enhance the solubility of CHL in an aqueous environment but not so strong to exert detrimental effects on its *in vitro* activity against Gram-positive and Gram-negative bacteria.

In summary, the findings from the present study support the possibility of complexing CHL with chemically modified β-cyclodextrins and of using such complexes to prepare aqueous eye drops with high antibiotic activity. These formulations could further increase the therapeutic benefits of CHL and provide an effective and low-cost treatment option of particular relevance to poor and developing countries, where ocular infections still represent a major health problem.

Experimental Section

Materials. Chloramphenicol ($C_{11}H_{12}NO_2O_5$) with a purity greater than 98%, Mueller-Hinton Agar 2 and Mueller-Hinton broth were purchased from Sigma-Aldrich (Milano, Italy). Pharmaceutical grade 2-hydroxypropyl-β-cyclodextrin (CAVASOL® W7-HP) and methyl-β-cyclodextrin (CAVASOL® W7-M) were obtained from Wacker Chemie AG (Burghausen, Germany). *E. coli* (ATCC 25922), *P. mirabilis* (ATCC 25933) and *S. aureus* (ATCC 25923) were supplied by KairoSafe (Duino Aurisina, Italy). *S. epidermidis* was isolated at "Tor Vergata" University Hospital (Rome, Italy).

Preparation of CHL-CD complexes and antibiotic eye drops. Inclusion complexes were prepared by wet kneading in the 1:1 molar ratio. Appropriate amounts of CDs were first wetted with distilled water and kneaded in an agate mortar for 10 min at room temperature. Then, a calculated amount of CHL was added into the wetted CDs and the mixture kneaded intensively for 30 min. The resulting solid dough was allowed to dry in air at room temperature to constant weight. Finally, the dry complexes were ground and sieved. Inclusion complex formation was confirmed by thermal analysis (DSC) and UV-difference spectroscopy.

CHL eye drops were prepared by dissolving the appropriate amount of the free or complexed drug in a commercial sterile isotonic solution for ophthalmic applications. Prior to being assayed, the formulations were passed through a 0.22-μm Millipore filter.

Phase-solubility studies. Experiments were carried out at 25 °C in stoppered glass tubes containing 10 mL of 0 to 150 mM cyclodextrins and an excess amount of CHL. The tubes were placed in a thermostated water bath (± 0.1 °C). Preliminary runs showed that 6 h were sufficient to reach equilibration. After this time, aliquots of the liquid were withdrawn, passed through a 0.45-μm nylon membrane filter and analysed spectrophotometrically at 278 nm.

Antimicrobial activity assay. Antimicrobial susceptibility tests were performed by the agar-well diffusion methods. Briefly, bacterial cells from an exponential-phase culture obtained from a single colony were spread on the surface of agar plates using a sterile swab soaked in the bacterial suspension. 9-mm wells were then cut in the agar and filled with 150 μL of a solution containing the free or complexed drug. After overnight incubation at 37 °C, the plates were examined and the diameters of the inhibition zones measured. Negative controls were carried out under the same assay conditions using drug-free HPβCD and MβCD as well as the ophthalmic solution alone. Measurements were made at least in duplicate and the results were averaged.

Acknowledgments. We greatly thank Prof. Antonio Scafuri ("Tor Vergata" University Hospital, Rome, Italy) for his contribution and Wacker Chemie AG (Burghausen, Germany) for the kind gift of cyclodextrin samples.

References

1. (a) Wareham, D. W.; Wilson, P. *J. Hosp. Med*. **2002**, *63*, 157-161. (b) Balbi, H. J. *Pediatr. Rev.* **2004**, *25*, 284-288.
2. (a) Fukuda, M.; Ohashi. H.; Matsumoto, S.; Mishima, S.; Shimomura, Y. *Cornea* **2002**, *21*, S86-S89. (b) Lam, R. F.; Lai, J. S. M.; Ng, J. S. K.; Rao, S. K.; Law, R. W. K.; Lam, D. S. C. *Hong Kong Med. J.* **2002**, *8*, 44-47. (c) Elton, M. *Pharm. J.* **2005**, *274*, 725-728.
3. Drainas, D.; Kalpaxis, D.; Coutsogeorgopoulos, C. *Eur. J. Biochem.* **1987**, *164*, 53-58.
4. (a) Ghate, D.; Edelhauser, H. F. *Expert Opin. Drug Deliv.* **2006**, *3*, 275-287. (b) Stella, V. J.; Nti-Addae, K. W. *Adv. Drug Deliv. Rev.* **2007**, *59*, 677-694.
5. (a) Brewster, M. E.; Loftsson, M. *Adv. Drug. Deliv. Rev.* **2007**, *59*, 645-666. (b) Arun, R.; Ashok Kumar, C. K.; Sravanthi, V. V. N. S. S. *Sci. Pharm.* **2008**, *76*, 567-598. (c) Chernykh, E. V.; Brichkin, S. B. *High Energy Chem.* **2010**, *44*, 83-100.
6. Loftssona, T.; Jarvinen, T. *Adv. Drug. Deliv. Rev.* **1999**, *36*, 59-79.
7. (a) Le Bourlais, C.; Acar, L.; Zia, H.; Sado, P. A.; Needham, T.; Leverge, R. *Prog. Retin. Eye Res.* **1998**, *1*, 33-58. (b) Stefánsson, E.; Loftsson, T. *J. Incl. Phenom. Macroc. Chem.* **2002**, *44*, 23-27.
8. (a) Zhang, J.; Wang, L.; Gao, C.; Zhang, L.; Xia, H. *J. Ocul. Parmacol. Ther.* **2008**, *24*, 501-506. (b) Valls, R.; Vega, E.; Garcia, M. L.; Egea, M. A.; Valls, J. O. *Open Med. Chem. J.* **2008**, *2*, 66-71. (c) Majumdar S.; Srirangam, R. *Pharm. Res.* **2009**, *26*, 1217-1225.
9. Higuchi, T.; Connors, K. A. *Adv. Anal. Chem. Instr.* **1965**, *4*, 117-212.
10. Chen, M.; Diao, G.; Zhang, E. *Chemosphere* **2006**, *63*, 522-529.
11. (a) Alfonso, E.; Crider, J. *Surv. Ophthalmol.* **2005**, *50*, S1-S6. (b) Kowalski, R. P.; Dhaliwal, D. K. *Expert Rev. Anti Infect. Ther.* **2005**, *3*, 131-139. (c) Woodward, M.; Randleman, J. B. *J. Cataract. Refract. Surg*. **2007**, *33*, 213-219.
12. (a) Sotozono, C.; Inagaki, K.; Fujita, A.; Koizumi, N.; Sano, Y.; Inatomi, T.; Kinoshita, S. *Cornea* **2002**, *21*, S94-S101. (b) Bourcier, T.; Thomas, F.; Borderie, V.; Chaumeil, C.; Laroche, L. *Br. J. Ophthalmol.* **2003**, *87*, 834-838.
13. Vuong, C.; Otto, M. *Microbes Infect.* **2002**, *4*, 481-489.