Carbohydrate Polymers 121 (2015) 320–327

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Inclusion complexes of chloramphenicol with β-cyclodextrin and aminoacids as a way to increase drug solubility and modulate ROS production

Virginia Aiassa [a,b], Ariana Zoppi [a,b], Inés Albesa [a], Marcela R. Longhi [a,b,*]

[a] Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina
[b] Unidad de Investigación y Desarrollo en Tecnología Farmacéutica (UNITEFA), CONICET, Universidad Nacional de Córdoba, Córdoba, Argentina

ARTICLE INFO

Article history:
Received 22 July 2014
Received in revised form 1 November 2014
Accepted 1 November 2014
Available online 8 December 2014

Keywords:
Chloramphenicol
Multicomponent complexes
Complex characterization
Solubility
ROS production
Microbiological activity

ABSTRACT

The aim of this study was to improve the solubility of chloramphenicol and reduce the production of reactive oxygen species (ROS) in leucocytes induced by this drug, using complexation. Multicomponent complexes were prepared by the addition of β-cyclodextrin with glycine or cysteine. Nuclear magnetic resonance and phase solubility studies provided information at the molecular level on the structure of the complexes and their association binding constants, respectively. In the solid state, all systems were extensively characterized by Fourier-transform infrared spectroscopy, scanning electron microscopy, thermal analysis and X-ray powder diffraction. Antimicrobial activity of inclusion complexes was investigated by agar diffusion methods. Finally ROS determination by chemiluminescence was used to investigate the effect of complex formation on the potential toxicity in human leucocytes. These studies revealed that multicomponent complexes can increase the aqueous solubility of chloramphenicol as well as reducing the stress by ROS production in leucocytes and maintaining its microbiological activity.

1. Introduction

Chloramphenicol (CP, Fig. 1A) is an antibacterial drug having a wide spectrum of activity against Gram positive and Gram negative cocci and bacilli, including anaerobes (Clinical Pharmacology Online, 2009; Sweetman, 2009). An idiosyncratic reaction resulting in aplastic anaemia can occur in predisposed patients that are unpredictable, irreversible, and frequently fatal, with an incidence of one case in 24,000 to 40,000 courses of therapy. This is thought to be due to the production by the gut flora of a nitro-reduction derivative of CP that can induce DNA damage in replicating haematopoietic stem cells, resulting in marrow hypocellularity and progressive pancytopaenia (Laporte, Vidal, Ballarin, & Ibanez, 1998). However, the most common presentation is a predictable dose-dependent toxicity resulting in an anaemia that is reversible upon drug withdrawal, which usually occurs when serum CP levels exceed 25 mg l^{-1} for prolonged periods of time. This is associated with the inhibition of mitochondrial protein synthesis, and is characterized by mild marrow hypocellularity, anaemia, neutropaenia and thrombocytopaenia (Walker et al., 1998). In addition as non-idiosyncratic aplastic anaemia and grey baby syndrome are dose-dependent complications of CP usage, these are non-haematopoietic toxicities of CP that, if left unrecognized and untreated, often result in cardiovascular collapse and death (Lam et al., 2002; Wiest, Cochran, & Tecklenburg, 2012).

These serious side effects and even fatal consequences limit the successful use of this drug. Several *in vitro* and *in vivo* results indicate that the toxicity of diverse chemotherapeutic drugs involves an increased production of reactive oxygen species (ROS) and oxidative stress (Butterfield, 2005). Research into the toxicology of CP indicates that its propensity can cause damage to blood-forming organs which might be related to its potential for nitro-reduction and subsequent production of nitric oxide. These observations suggest that the para-nitro group of CP could be the cause of haematotoxicity in susceptible people (Holt & Bajoria, 1999).

Abbreviations: ROS, reactive oxygen species; CP, chloramphenicol; CPMC, chloramphenicol multicomponent complexes; Cys, cysteine; Gly, glycine; CD, cyclodextrins; CL, chemiluminescence; PM, physical mixture; FD, freeze-drying; FT-IR, Fourier-transform infrared spectroscopy; SEM, scanning electron microscopy; DSC, differential scanning calorimetry; TG, thermogravimetric analysis; PXRD, powder X-ray diffraction; AA, amino acid.

* Corresponding author at: Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina. Tel.: +54 0351 5353865; fax: +54 0351 5353865x53364.
E-mail addresses: aiassa@fcq.unc.edu.ar (V. Aiassa), ariana@fcq.unc.edu.ar (A. Zoppi), inesalbesa@fcq.unc.edu.ar (I. Albesa), mrlcor@fcq.unc.edu.ar, mrlonghi@gmail.com (M.R. Longhi).

http://dx.doi.org/10.1016/j.carbpol.2014.11.017
0144-8617/

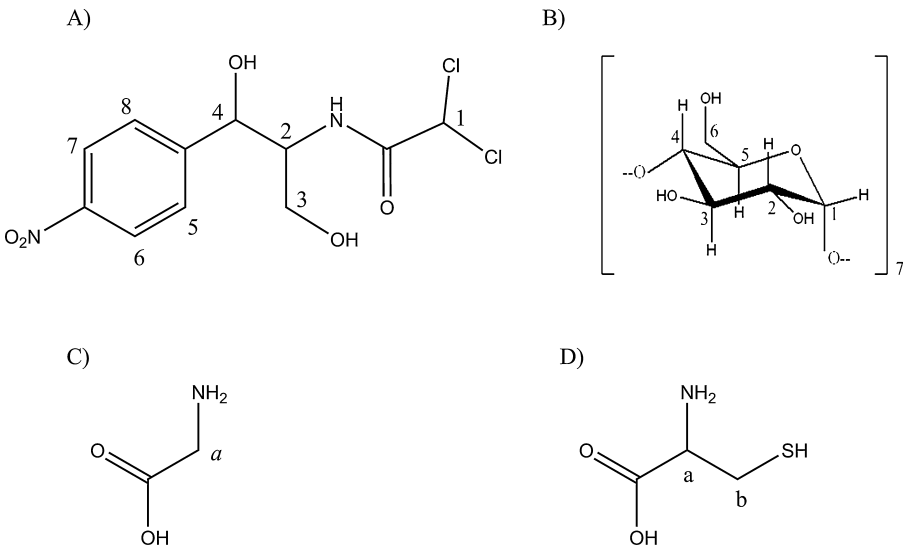

Fig. 1. Chemical structure and proton atom numbering scheme of: (A) CP, (B) βCD, (C) Gly and (D) Cys.

In addition, CP is slightly soluble in water and has a bitter taste, Shi and Zhou (2011) demonstrated that CP complexation with cyclodextrins (CD) enhances its solubility and covers up its bitter taste. CD are in fact water-soluble cyclic oligosaccharides composed of 6 (α-CD), 7 (β-CD, Fig. 1B) or 8 (γ-CD) D-(+)-glucopyranose units arranged in a truncated cone-shaped structure. Different molecules can penetrate into the relatively hydrophobic cavity and form non-covalent inclusion complexes, thus modifying their physical, chemical and biological properties (Loftsson & Brewster, 2010, 2012).

Different researchers have reported the preparation and characterization of CP complexes with cyclodextrins (Hirayama, Usami, Kimura, & Uekama, 1997; Mashhood Ali, Asmat, & Maheshwari, 2004; Li, Luo, & Liu, 2005; Fatiha, Leila, Eddine, & Leila, 2013; Ramos et al., 2013) and demonstrated that the inclusion complexes are an effective way to increase the solubility of CP (Zuorro, Fidaleo, & Lavecchia, 2010; Shi & Zhou, 2011).

In addition, in recent years the use of the formation of multicomponent complexes with cyclodextrin and a third auxiliary substance has become more frequent in order to facilitate the therapeutic application of different drugs (Palma et al., 2009; Mora, Longhi, & Granero, 2010; Garnero & Longhi, 2010; De Melo et al., 2013; Taupitz, Dressman, Buchanan, & Klein, 2013; Wang et al., 2013; Chadha et al., 2014). This approach permits a lower dose of cyclodextrins to be used and increases the complexation efficiency. Moreover, by specifically selecting of the auxiliary substance added to the multicomponent complex, it is possible to reduce other problematic behaviours of the drugs such as their toxicological properties.

It is known that thiol compounds such as cysteine (Cys, Fig. 1C) and reduced glutathione (GSH) play an important role in human aging and age related diseases. In fact, Cys supplementation has been shown by antioxidant actions to ameliorate several parameters which are known to degenerate during human aging. The decline in Cys efflux during aging observed in erythrocytes is an important factor in the development of oxidative stress (Kumar & Maurya, 2013). Also, a protective role has been suggested for glycine (Gly, Fig. 1D) action against the anoxic and oxidative stress produced by toxic agents (Qu, Ikejima, Zhong, Waalkes, & Thurman, 2002).

The present work was carried out with the purpose of evaluating the effect of CP-βCD-antioxidant complexation on CP aqueous solubility, antimicrobial activity and oxidative stress production in human leucocytes. CP multicomponent complexes with βCD and antioxidants (Cys or Gly) were prepared in solution and in solid state with the effects of complexation on drug solubility, affinity constant (K_C) and stoichiometry for the complexes being determined in aqueous solution, by means of phase-solubility studies and by ^{1}H NMR and 2D NMR spectroscopy. In the solid state, the multicomponent systems, prepared by means of a simple physical mixture (PM) or by freeze-drying (FD) methods, were studied by Fourier-transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), thermogravimetric analysis (TG) and powder X-ray diffraction (PXRD). The antimicrobial activity of inclusion complexes was investigated by the agar diffusion methods against the Gram-positive species *Staphylococcus aureus* and the Gram-negative species *Escherichia coli* and *Pseudomonas aeruginosa*. Finally, the potential toxic effects on human leucocytes were evaluated by using assays of ROS determination by chemiluminescence (CL).

2. Materials and methods

2.1. Chemicals and reagents

CP base and Gly were obtained from Parafarm (Argentina). βCD (MW = 1135) was kindly supplied by Roquette (France). Cys, Ficoll–Hypaque (Histopaque-1077), dextran, luminol (4-amino-2,3-dihydro-1,4-phthalazine-dione) and the D_2O 99.9 atom % *D* used in spectroscopic studies were purchased from Sigma®. All other materials and solvents were of analytical reagent grade. A Milli-Q Water Purification System (Millipore, USA) generated the water used in these studies.

2.2. Phase solubility analysis

Experiments were carried out at 25 °C in stoppered glass tubes containing an excess amount of CP (100 mg) with Cys or Gly 10 mM, and 5 ml of βCD with increasing concentrations (0–13.2 mM), according to the method reported by Higuchi and Connors (1965). The tubes were placed in a 25.0 (±0.1) °C thermostatized water bath (Circulators HAAKE F3-K, Germany) for 72 h, and the resulting suspensions were vortexed 15 s twice a day to achieve equilibrium. Each sample suspension was filtered through a 0.45 μm membrane, and the filtrate was appropriately diluted for quantitative analysis of CP in each multicomponent complex by the HPLC method.

The HPLC equipment consisted of an Agilent S1100 system with UV detection at 278 nm, using a Luna C8 (4.6 × 150 mm, 5 μm, Phenomenex, USA) reversed-phase column with pre-column. The mobile phase was acetonitrile used with a pH 3.0 potassium phosphate buffer (10 mM) at a 50:50 mixture, and a flow rate of 1.0 ml min^{-1}. Assays were performed at 25 °C, by injecting 10 μl of solution in each chromatographic run. Under these conditions, the retention time for CP was 4.4 min Each experiment was repeated at least three times, and the results reported are the mean values. The stability constant values for the corresponding complexes were calculated from the slope of the phase-solubility diagrams and S_0, according to the following equation:

$$K_C = \frac{\text{slope}}{S_0\,(1 - \text{slope})} \quad (1)$$

The stability of the drug was determined in water at 25 °C, with no drug degradation being found after 72 h of incubation.

2.3. NMR studies

The NMR spectra of multicomponent systems were recorded on a Bruker Avance II high resolution spectrometer equipped with a broad band inverse probe (BBI) and a variable temperature unit (VTU). The spectra were measured at 298 K. D_2O was used as solvent and the resonance at 4.8 ppm due to the residual solvent (HDO) was used as the internal reference. Induced changes in the ^1H chemical shifts for CP, βCD, Gly and Cys ($\Delta\delta$), which originated due to their complexations, were calculated using the following equation:

$$\Delta\delta = \delta_{\text{complex}} - \delta_{\text{free}} \quad (2)$$

The 2D-ROESY spectra of the multicomponent systems were recorded on the same spectrometer, with a relaxation delay of 2 s and 32 scans.

2.4. Preparation of complexes in solid state

The preparation of solid CP multicomponent systems with a 1:1:1 molar ratio was carried out using the freeze-drying (FD) method. Solutions of CP with βCD and Cys or Gly were prepared in water, frozen at −40 °C and then freeze dried (Freeze Dri 4.5 Labconco Corp., Kansas City, MI). Physical mixtures (PM) were prepared by mixing CP with βCD and Cys or Gly powders, uniformly in a mortar at a 1:1:1 molar ratio.

2.5. Fourier-transform infrared spectroscopy (FT-IR)

FT-IR spectra of CP, βCD, Cys, Gly and their complexes (KBr disks) were recorded on a Nicolet Avatar 360 FT-IR spectrometer. The spectra of the complexes were compared with those of the corresponding 1:1:1 molar ratio physical mixture and also with the pure CP, βCD, Cys and Gly. All spectra were obtained and processed using EZ OMNIC E.S.P v.5.1 software.

2.6. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TG)

The DSC curves of the CP, βCD, Cys, Gly and their complexes were produced using a DSC TA 2920, and the TG curves were recorded on a TG TA 2920 (TA Instruments, Inc., New Castle, DE). The samples were placed in aluminium hermetic pans, with the experiments being carried out under a nitrogen gas flow, at a heating rate of 10 °C min^{-1}, and over a temperature range of 25–300 °C and 25–350 °C for the DSC and TG studies, respectively. In both cases, data were obtained and processed using TA Instruments Universal Analysis 2000 software.

2.7. Powder X-ray diffraction studies

The powder X-ray diffraction patterns were obtained with a Philips X'Pert PRO PAN analytical powder diffractometer (Philips®, The Netherlands), using Ni-filtered Cu $K\alpha$ radiation at 45 kW and 30 mA The diffractograms were recorded from 2° to 40° (2θ), with a step size of 0.02° and a scan step time of 2 s.

2.8. Scanning electron microscopy studies (SEM)

Microscopic morphological structures of the solid samples were investigated and photographed using a Carl Zeiss Σigma scanning electron microscope, at the Laboratorio de Microscopía y Análisis por Rayos X (LAMARX) of the National University of Córdoba. The samples were fixed on a brass stub using double-sided aluminium tape, and to improve the conductivity, they were gold/palladium-coated under vacuum employing a sputter coater Quorum 150. The magnification selected was sufficient to appreciate in detail the general morphology of the samples under study.

2.9. Antimicrobial activity assay

Antimicrobial susceptibility tests were performed by the agar diffusion method with some modifications (Zuorro et al., 2010). Briefly, American Type Culture Collection (ATCC®) bacterial cells of *S. aureus* ATCC® 25923, *E. coli* ATCC® 25922 or *P. aeruginosa* ATCC® 27853 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 diluted to 10^6 colony forming units per millilitres (CFU)ml^{-1}. A disk of 6 mm of sterile Whatman N° 1 membrane filters resting on an agar culture medium was inoculated with 10 μl of a solution containing the free or complexed CP (30 μg). Aqueous solutions of pure βCD, Cys and Gly were used as a placebo control. After overnight incubation was performed at 37 °C, the plates were examined and the diameters of the inhibition zones measured. Measurements were made in triplicate and the results were averaged.

2.10. Leukocyte preparation from human blood

Leucocytes were isolated by a combined dextran (Sigma, average M_W = 78.000)/Ficoll–Hypaque (Histopaque-1077, Sigma) sedimentation procedure. Sedimentation in 6% dextran solution was performed before carrying out gradient centrifugation. A mixture of Ficoll–Hypaque was then used to isolate the mononuclear cells from the remaining haematic cells. After sedimentation, hypotonic lyses of the erythrocytes were performed. The leukocyte layer was washed twice and suspended in Hanks' balanced salt solution. Cell preparation was adjusted to 10^6 ml^{-1} leucocytes for the assay.

2.11. ROS determination by the chemiluminescence (CL) assay

CL was measured at room temperature using a luminometer (Bio-Orbit 1253, Turku, Finland) with disposable polypropylene tubes. The basal value of the leucocytes CL was measured in the presence of luminol (4-amino-2,3-dihydro-1,4-phthalazine-dione, Sigma). A volume of 0.2 ml of 10^6 ml^{-1} leucocytes was incubated with 0.2 ml of 3.4 μM luminol and: (1) 0.2 ml of CP (0.3 mM), (2) 0.2 ml of βCD (0.3 mM), (3) 0.2 ml of CP:βCD (0.3 mM of CP, 0.3 mM of βCD), (4) 0.2 ml of CP:βCD:Gly (0.3 mM of CP, 0.3 mM of βCD, 10 mM of Gly) or (5) 0.2 ml of CP:βCD:Cys (0.3 mM of CP, 0.3 mM of βCD, 10 mM of Cys).

The CL background of each vial was checked before being used and the light emission was measured for 105 s, at 5 s intervals. Results were expressed in relative light units (RLU) at different

times, with the background value being subtracted (Caldefie-Chezet et al., 2002).

2.12. Statistical analysis

Data from the ROS determination were statistically assessed by a one-way ANOVA. Differences were considered to be significant at $P < 0.05$.

3. Results and discussion

3.1. Behaviour of the multicomponent systems in aqueous solution

3.1.1. Phase solubility analysis

The phase solubility profiles of CP with βCD and Gly or Cys (data not shown) can be classified as A_L being of the type according to Higuchi and Connors (1965), and showed a linear increase of drug solubility, which is indicative of the formation of soluble complexes.

The corresponding stability constant values calculated from each phase-solubility diagram were 107 ± 4 and $101 \pm 8\ M^{-1}$ for the systems with Gly and Cys, respectively. In both cases, the increase in drug solubility was 1.5 fold. From these values, it can be deduced that these were similar interactions between the drug and the βCD with each amino acid. The stability constant of the binary complex CP:βCD was also calculated for comparison, obtaining a value of $180 \pm 12\ M^{-1}$, which is comparable to that found for this system for Hirayama et al. (1997). So, it is possible to suggest that the differences found for the stability constants of the multicomponent complexes regarding with the binary one may be due to the amino acid that competes with the drug on the formation of hydrogen bonds with the macromolecule.

3.1.2. NMR studies

Table 1 presents the assignments of the CP, βCD, Gly and Cys peaks and the chemical shift deviations due to complexation (proton numbering in Fig. 1). By comparing the chemical shifts of the CP:βCD:AA (AA = amino acid) systems with those of the pure components, it is possible to observe significant changes that are indicative of the complex formation between CP and βCD in the presence of each AA.

First, the CP signals show a considerable deshielding as a result of the interaction with βCD and/or each amino acid. Second, the βCD signals are also affected, revealing in this case a marked shielding of the signals corresponding to the H_3 and H_5 protons, thus suggesting the insertion of the aromatic ring of the drug into the inside the macromolecule.

In order to confirm the interaction mode of these systems, the ROESY spectra (Fig. 2) were examined, with intermolecular cross-peaks being observed in these assays between the aromatic protons of CP (H_5–H_8 and H_7–H_6) and the protons located inside the βCD cavity (H_3 and H_5). From these results, we can confirm the formation of inclusion complexes for the two systems studied, which had similar conformations in aqueous solution, characterized by a deep insertion of the aromatic ring of CP into the hydrophobic cavity of βCD. Finally, the results obtained for the binary system CP:βCD (Table 1) were compared with those of the ternary ones, with differences being observed in the displacement of the chemical shifts in the presence of the third component. Additionally, is important to note that the protons of both amino acids also experienced shifts in the CP:βCD:AA complexes. All these observation, allows us to hypothesize that Gly and Cys are interacting with the inclusion complex, but without substantially modifying the CP insertion mode.

3.2. Behaviour of the multicomponent systems in solid state

A comprehensive multicomponent system characterization, in solid state, was performed by applying different analytical techniques, including: FT-IR (Fig. 3), DSC and TG (Fig. 4), PXRD (Fig. 5) and SEM (Fig. 6). In addition, the behavior of the pure components was compared with the multicomponent systems obtained by using the PM or FD methods. Below, we sum up the most relevant results obtained by this characterization.

Notably, for all of the analytical techniques used, it can be concluded that in the PM systems the natural structure of each pure component remained revealing that no interactions took place among them. In contrast, the first evidence of complex formations in the FD systems prepared with Gly or Cys was obtained by the FT-IR studies, with the most interesting feature being the modification of the CP bands at 1686 cm^{-1} (assigned to C=O vibration) and 1559 cm^{-1} (corresponding to NO_2 group). In the CP:βCD:Gly system, the band at 1686 cm^{-1} was shifted to 1689 cm^{-1}, while in the CP:βCD:Cys system the bands at 1686 and 1559 cm^{-1} decreased notably. The modification of these bands may suggest the establishment of intermolecular interactions involving these groups, thus indicating the formation of a multicomponent complex.

Fig. 4 shows the TG and DSC curves of the pure components and multicomponent systems. The TG results for the FD systems, in both

Table 1
Chemical shifts displacements for the protons CP, βCD, Gly and Cys in complex forms.

System	CP protons	Δδ (ppm)	AA protons	Δδ (ppm)	βCD protons	Δδ (ppm)
CP:βCD	H_1	−0.0361	–	–	H_1	−0.0508
	H_2	0.1023			H_2	−0.0366
	H_3	Overlapped			H_3	−0.1068
	H_4	0.0962			H_4	−0.0419
	H_5–H_8	0.0891			H_5	−0.184
	H_7–H_6	−0.0489			H_6	−0.0429
CP:βCD:Gly	H_1	0.0098	H_a	0.0097	H_1	0.3831
	H_2	0.1325			H_2	0.0052
	H_3	Overlapped			H_3	−0.077
	H_4	0.1272			H_4	−0.0006
	H_5–H_8	0.1270			H_5	−0.1196
	H_7–H_6	−0.0019			H_6	0.0009
CP:βCD:Cys	H_1	0.0149	H_a	0.0176	H_1	−0.0067
	H_2	0.1094	H_b	−0.1297	H_2	0.0031
	H_3	Overlapped			H_3	−0.0612
	H_4	0.1055			H_4	−0.0016
	H_5–H_8	0.1060			H_5	−0.1010
	H_7–H_6	0.0061			H_6	0.0023

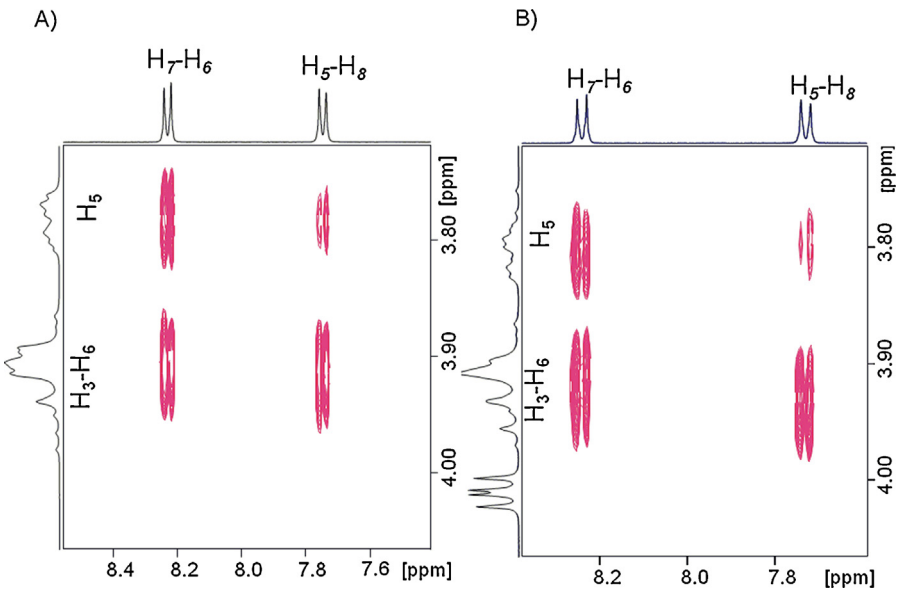

Fig. 2. Partial contour plot of the 2D ROESY spectrum of: (A) CP:βCD:Gly system and (B) CP:βCD:Cys system.

cases, showed that the onset temperature for thermal decomposition of CP shifted to a lower temperature, probably due to complex formation. In addition, the DSC curves of pure CP presented a characteristic melting endothermic peak at 152 °C, whereas the FD systems did not show any endothermic peak at this temperature, indicating that amorphous solids were obtained in both these cases.

These thermal analysis results are supported by findings from the PXRD measurements (Fig. 5), since the extremely low

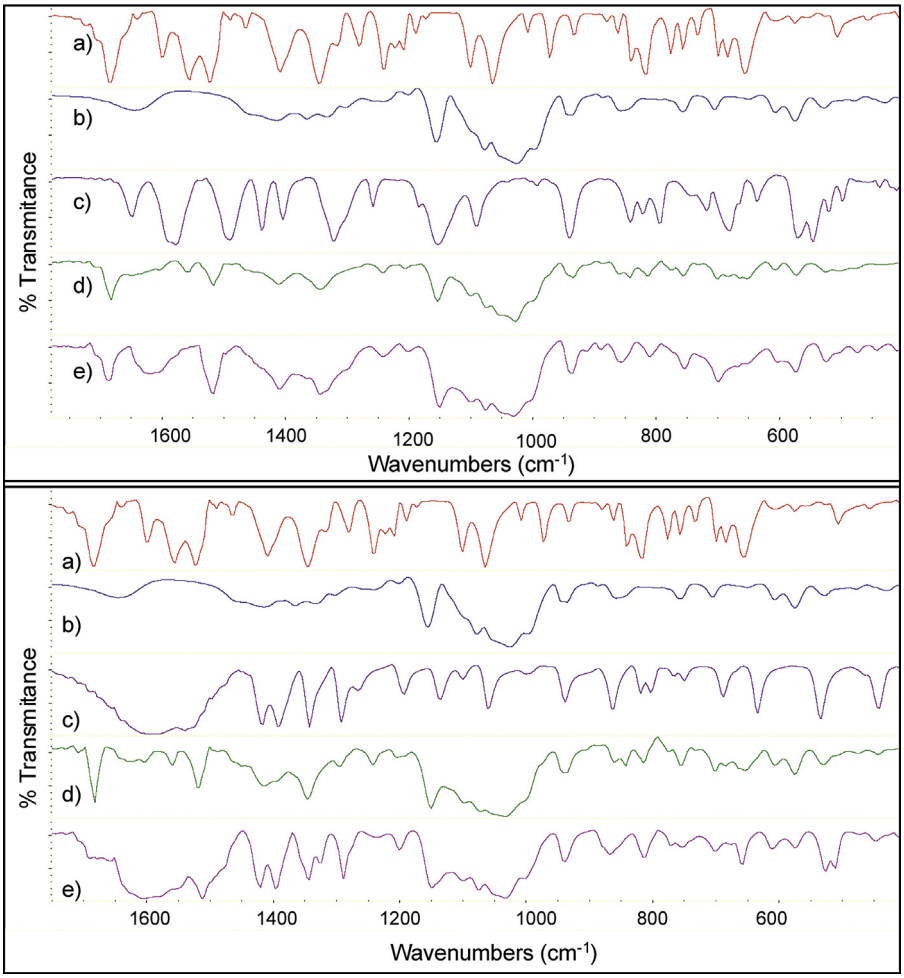

Fig. 3. IR spectra of: (A) (a) CP, (b) βCD, (c) Gly, (d) CP:βCD:Gly PM system and (e) CP:βCD:Gly FD system, (B) (a) CP, (b) βCD, (c) Cys, (d) CP:βCD:Cys PM system and (e) CP:βCD:Cys FD system.

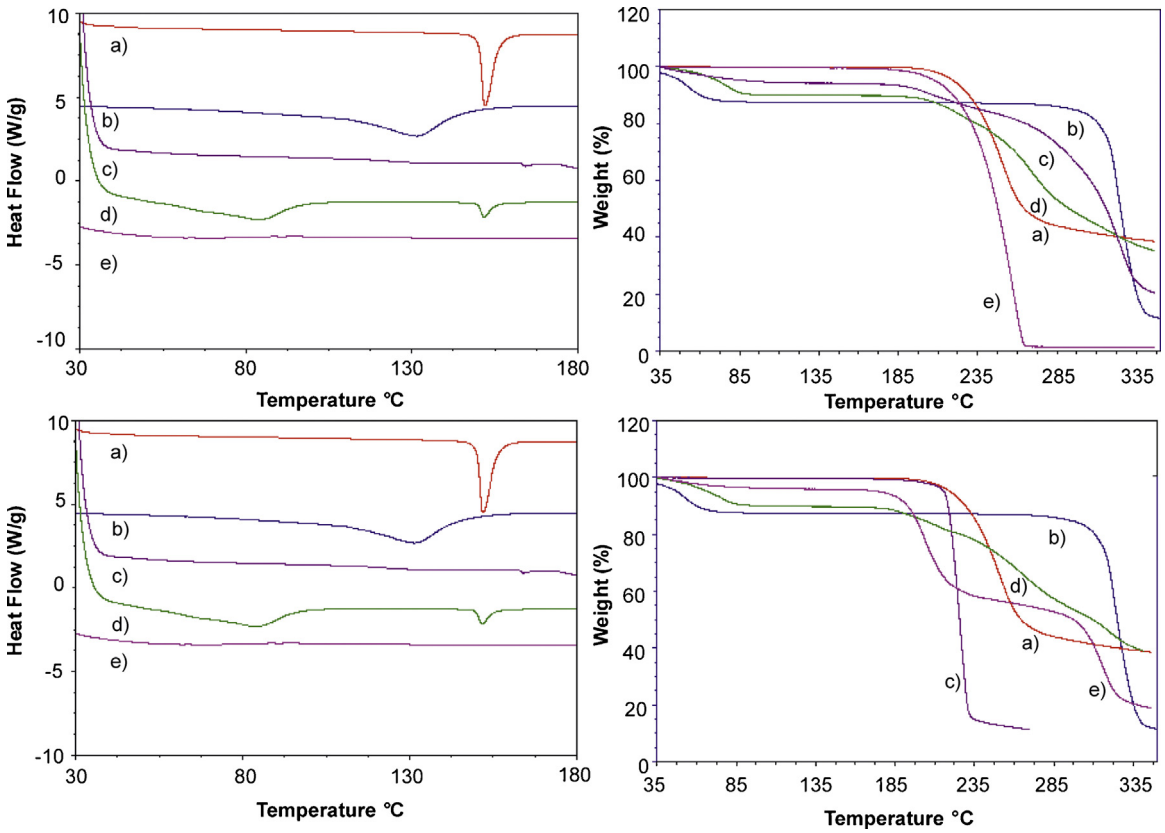

Fig. 4. TG and DSC curves of: (A) (a) CP, (b) βCD, (c) Gly, (d) CP:βCD:Gly PM system and (e) CP:βCD:Gly FD system, (B) (a) CP, (b) βCD, (c) Cys, (d) CP:βCD:Cys PM systems and (e) CP:βCD:Cys FD system.

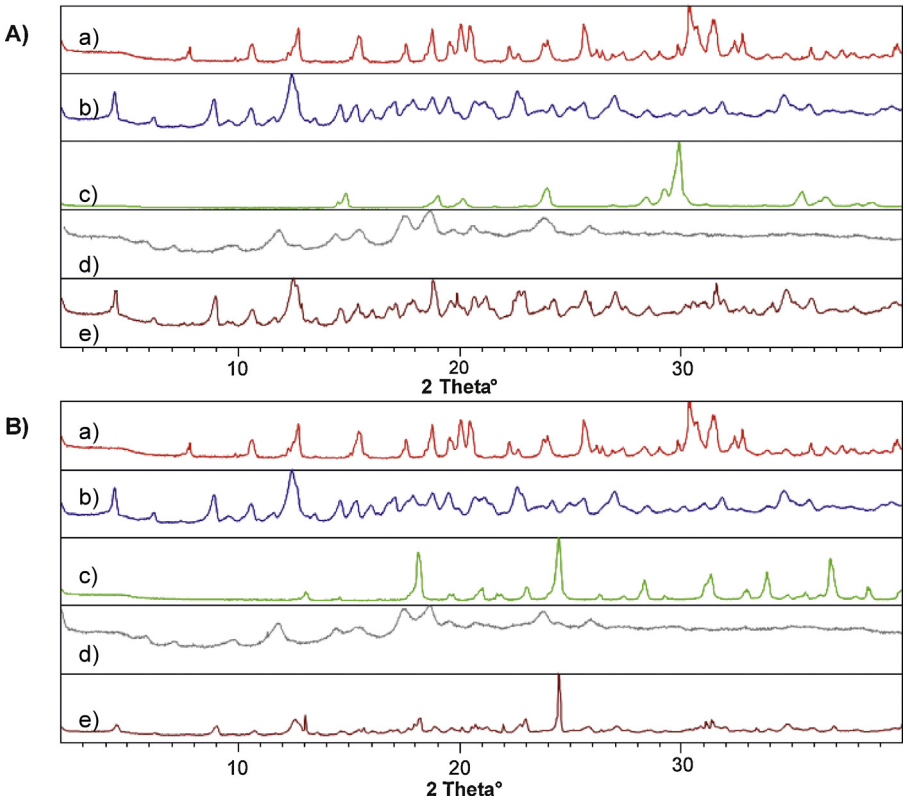

Fig. 5. X-ray diffractograms of: (A) (a) CP, (b) βCD, (c) Gly, (d) CP:βCD:Gly FD system and (e) CP:βCD:Gly PM system, (B) (a) CP, (b) βCD, (c) Cys, (d) CP:βCD:Cys FD systems and (e) CP:βCD:Cys PM system.

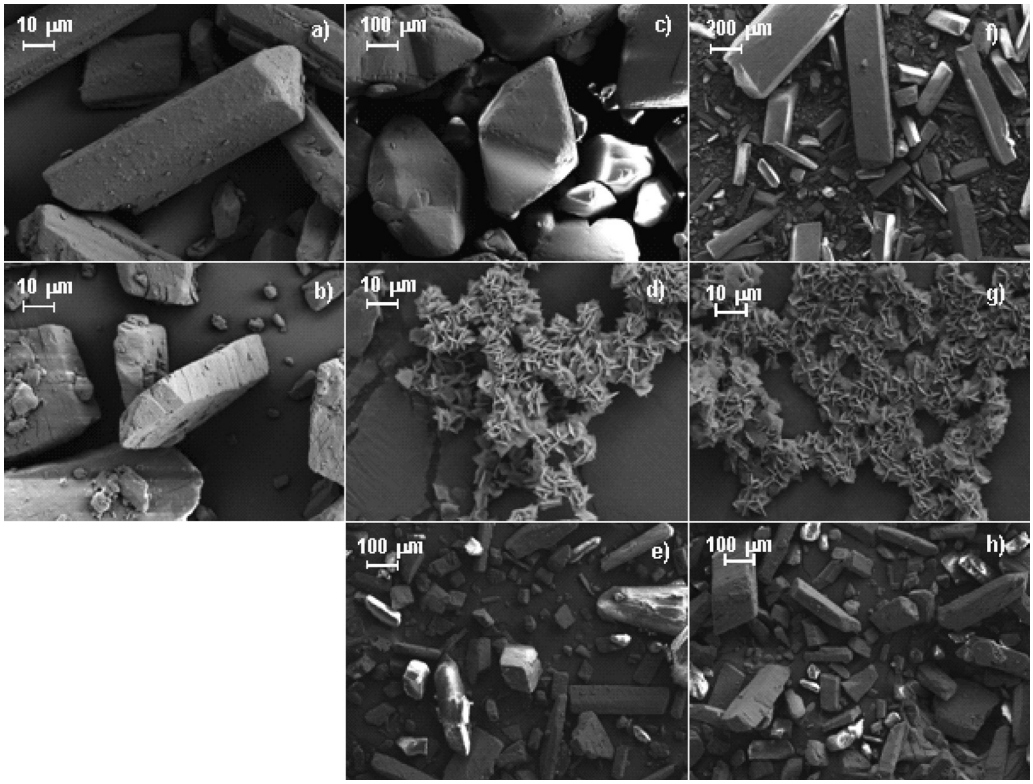

Fig. 6. Scanning electron microphotographs of: (a) CP, (b) βCD, (c) Gly, (d) CP:βCD:Gly FD system, (e) CP:βCD:Gly PM system, (f) Cys, (g) CP:βCD:Cys FD system, (h) CP:βCD:Cys PM system.

crystalinity of the FD multicomponent systems suggests the complete amorphization of CP.

Finally, the SEM images of the FD samples (Fig. 6) showed similar morphologies, which were completely different to those of the pure components. Taken together, all these experiments confirm the formation of amorphous multicomponent complexes between CP and βCD, when using Gly or Cys as a third component, for systems prepared by the FD method.

3.3. Antimicrobial activity

The antimicrobial activity of the multicomponent systems was studied in order to assess the ability of βCD to release the drug from the inclusion complex, taking into account that a decrease in the drug release capability would in turn cause a reduction in the antimicrobial potency of the CP present in a given pharmaceutical formulation. The antimicrobial activity of CP multicomponent complex against *S. aureus*, *E. coli* and *P. aeruginosa* was investigated by the agar diffusion methods and compared with that of pure CP. These assays showed that there were no significant differences between the pure and complexed inhibition zones of CP, for the three species under study. The inhibition zones were 24.5 ± 0.7 mm, 25 ± 1 mm, 24.5 ± 0.7 mm, 25.5 ± 0.5 mm for CP, CP:βCD, CP:βCD:Cys and CP:βCD:Gly respectively in *S. aureus*, 25 ± 1 mm for CP, CP:βCD and both multicomponent complexes in *E. coli* and 25.6 ± 0.6 mm, 26 ± 1 mm, 26 ± 1 mm and 28 ± 1 mm for CP, CP:βCD, CP:βCD:Cys and CP:βCD:Gly respectively in *P. aeruginosa*. These results are indicating that drug complexation did not interfere with the microbiological activity of CP and allow us to postulate that the interactions between the components in the complex were sufficient to improve the solubility of the drug though not so strong as to reduce its microbiological activity. Furthermore, in the same test, the use of βCD, Cys and Gly alone did not present any antimicrobial activity against the species under study.

3.4. ROS determination

Determining the capacity of CP to generate aplastic anaemia requires evaluation of the metabolic alterations that precede this severe affection. Thus, the development of toxicity assays could be useful in ameliorating this severe risk. Previous studies have demonstrated that ROS and nitrite production, together with the alteration of antioxidant enzymes, may explain the leukotoxicity of CP in neutrophils (Páez, Becerra, & Albesa, 2008). In fact, is known that cells contain some antioxidant systems in order to protect themselves from the injury induced by increased intracellular ROS. Several investigations have reported that the levels of GSH are modified in response to anaesthetics and other drugs (Wong et al., 2006; Fernández et al., 2004). Consequently, it could be interesting to determine the effect of exogenous antioxidants on oxidative stress caused by CP, especially as the effect of CP combined with antioxidants has not yet been investigated.

Therefore, in the present study the amino acids Gly and Cys were used as a third component in the multicomponent complexes, taking into consideration that these two amino acids have been previously proposed as antioxidants and cytoprotectors (Deters, Strubelt, & Younes, 1997; Kumar & Maurya, 2013). Now, recalling that the haematological alteration after therapy with CP seemed to be a consequence of oxidative damage, the results effectively confirm the presence of oxidative stress of leukocytes produced by CP, since ROS production measured by CL (Fig. 7) was significantly greater in leucocytes treated with CP (100 μg/ml) in comparison with the untreated ones ($P < 0.05$). βCD alone did not produce an increase in ROS, while the CP:βCD complex behaved as pure CP and showed a rise in ROS. Finally when the multicomponent systems were assayed, the production of ROS was significantly lower than in the untreated samples, suggesting that Gly and Cys acted as potent ROS inhibitor/scavengers, thereby indicating the advantages of these multicomponent formulations containing AA in order

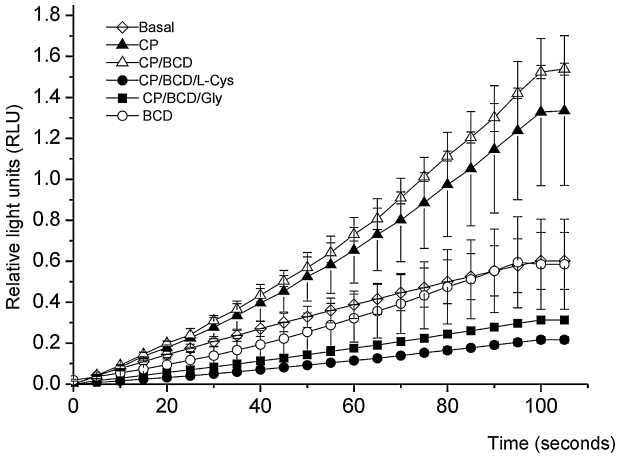

Fig. 7. Reactive oxygen species induced by Chloramphenicol quantification in human leucocytes by chemiluminescence assay. The graph shows the treatment for 105 s with CP (▲), βCD (○), CP:βCD (△), CP:βCD:Gly (■) and CP:βCD:Cys (●). For both ternary systems, $P < 0.05$, compared to the untreated sample (◊).

to reduce the harmful effects of CP without affecting its therapeutic activity.

4. Conclusions

This study has shown that the solubility of CP can be improved by inclusion complexation with CD and AA, with the complexation of this antimicrobial drug not interfering with its microbiological activity. Moreover, the antioxidants Gly and Cys when used as a third component were able to produce a protective effect against the increase of ROS induced by CP in leucocytes. Based on this behaviour of the multicomponent complexes, it can be concluded that they are potentially useful application in the future formulation of dosage forms of chemotherapeutic drugs that produce ROS and oxidative stress.

Acknowledgments

The authors thank the Secretaría de Ciencia y Técnica de la Universidad Nacional de Córdoba (SECyT) and the Consejo Nacional de Investigaciones Científicas y Tecnológicas de la Nación (CONICET) for financial support. We also thank Ferromet S.A. (agent of Roquette in Argentina) for its donation of β-cyclodextrin. We are grateful to Dr. Gloria Bonetto for NMR measurements and for her helpful discussions of the ^{1}H NMR spectra, and to Dr. Paul Hobson, native English speaker, for revision of the manuscript.

References

Butterfield, D. A. (2005). Free radical mediated oxidative stress and toxic side effects in brain induced by the anti-cancer drug adriamycin: Insight into chemobrain. *Free Radical Research*, *39*, 1147–1154.

Caldefie-Chezet, F., Walrand, S., Moinard, C., Tridon, A., Chassagne, J., & Vasson, M. P. (2002). Is the neutrophil reactive oxygen species production measured by luminol and lucigenin chemiluminescence intra or extracellular? Comparison with DCFH-DA flow cytometry and cytochrome c reduction. *Clinica Chimica Acta*, *319*, 9–17.

Chadha, R., Bala, M., Arora, P., Jain, D. V. S., Pissurlenkar, R. R. S., & Coutinho, E. C. (2014). Valsartan inclusion by methyl-β-cyclodextrin: Thermodynamics, molecular modelling, Tween 80 effect and evaluation. *Carbohydrate Polymers*, *103*, 300–309.

Clinical Pharmacology Online. (2009). *Gold standard*. Tampa, FL: Elsevier Company. Available from ⟨www.clinicalpharmacology.com⟩ (cited February 13, 2013).

Deters, M., Strubelt, O., & Younes, M. (1997). Protection by glycine against hypoxia–reoxygenation induced hepatic injury. *Research Communications in Molecular Pathology and Pharmacology*, *97*, 199–213.

De Melo, P. N., Barbosa, E. G., De Caland, L. B., Carpegianni, H., Garnero, C., Longhi, M., et al. (2013). Host-guest interactions between benznidazole and beta-cyclodextrin in multicomponent complex systems involving hydrophilic polymers and triethanolamine in aqueous solution. *Journal of Molecular Liquids*, *186*, 147–156.

Fatiha, M., Leila, L., Eddine, K. D., & Leila, N. (2013). Computational investigation of enol/keto chloramphenicol with β-cyclodextrin. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *77*(1–4), 421–427.

Fernández, C., Ramos, A. M., Sancho, P., Amrán, D., de Blas, E., & Aller, P. (2004). 12-*O*-Tetradecanoylphorbol-13-acetate may both potentiate and decrease the generation of apoptosis by the antileukemic agent arsenic trioxide in human promonocytic cells. Regulation by extracellular signal-regulated protein kinases and glutathione. *Journal of Biological Chemistry*, *279*, 3877–3884.

Higuchi, T., & Connors, K. A. (1965). Phase-solubility techniques. In C. N. Reilly (Ed.), *Advances in analytical chemistry and instrumentation* (pp. 117–212). New York, NY: Wiley-Interscience.

Hirayama, F., Usami, M., Kimura, K., & Uekama, K. (1997). Crystallization and polymorphic transition behavior of chloramphenicol palmitate in 2-hydroxypropyl-β-cyclodextrin matrix. *European Journal of Pharmaceutical Sciences*, *5*(1), 23–30.

Holt, D. E., & Bajoria, R. (1999). The role of nitro-reduction and nitric oxide in the toxicity of chloramphenicol. *Human & Experimental Toxicology*, *18*, 111–118.

Garnero, C., & Longhi, M. (2010). Development of HPLC and UV spectrophotometric methods for the determination of ascorbic acid using hydroxypropyl-β-cyclodextrin and triethanolamine as photostabilizing agents. *Analytica Chimica Acta*, *659*, 159–166.

Kumar, P., & Maurya, P. K. (2013). L-Cysteine efflux in erythrocytes as a function of human age: Correlation with reduced glutathione and total antioxidant potential. *Rejuvenation Research*, *16*, 179–184.

Lam, R. F., Lai, J. S. M., Ng, J. S. K., Rao, S. K., Law, R. W. K., & Lam, D. S. C. (2002). Topical chloramphenicol for eye infections. *Hong Kong Medical Journal*, *8*, 44–47.

Laporte, J. R., Vidal, X., Ballarin, E., & Ibanez, L. (1998). Possible association between ocular chloramphenicol and aplastic anaemia—The absolute risk is very low. *British Journal of Clinical Pharmacology*, *46*, 181–184.

Li, N. B., Luo, H. Q., & Liu, S. P. (2005). Resonance Rayleigh scattering study of the inclusion complexation of chloramphenicol with β-cyclodextrin. *Talanta*, *66*(2 Spec. Iss.), 495–500.

Loftsson, T., & Brewster, M. E. (2010). Pharmaceutical applications of cyclodextrins: Basic science and product development. *The Journal of pharmacy and pharmacology*, *62*, 1607–1621.

Loftsson, T., & Brewster, M. E. (2012). Cyclodextrins as functional excipients: Methods to enhance complexation efficiency. *Journal of Pharmaceutical Science*, *101*, 3019–3032.

Mashhood Ali, S., Asmat, F., & Maheshwari, A. (2004). NMR spectroscopy of inclusion complex of D-(−)-chloramphenicol with β-cyclodextrin in aqueous solution. *Farmaco*, *59*(10), 835–838.

Mora, M. J., Longhi, M. R., & Granero, G. E. (2010). Synthesis and characterization of binary and ternary complexes of diclofenac with a methyl-β-CD and monoethanolamine and in vitro transdermal evaluation. *European Journal of Medicinal Chemistry*, *45*, 4079–4088.

Páez, P. L., Becerra, M. C., & Albesa, I. (2008). Chloramphenicol-induced oxidative stress in human neutrophils. *Basic & Clinical Pharmacology & Toxicology*, *103*, 349–353.

Palma, S. D., Tártara, L. I., Quinteros, D., Allemandi, D. A., Longhi, M. R., & Granero, G. E. (2009). An efficient ternary complex of acetazolamide with HP-β-CD and TEA for topical ocular administration. *Journal of Controlled Release*, *138*, 24–31.

Qu, W., Ikejima, K., Zhong, Z., Waalkes, P., & Thurman, R. (2002). Glycine blocks the increase in intracellular free Ca^{2+} due to vasoactive mediators in hepatic parenchymal cell. *American Journal of Physiology, Gastrointestinal and Liver Physiology*, *283*, G1249–G1256.

Ramos, A. I., Braga, T. M., Silva, P., Fernandes, J. A., Ribeiro-Claro, P., De Fátima Silva Lopes, M., et al. (2013). Chloramphenicol cyclodextrin inclusion compounds: Co-dissolution and mechanochemical preparations and antibacterial action. *CrystEngComm*, *15*(15), 2822–2834.

Shi, J. H., & Zhou, Y. F. (2011). Inclusion interaction of chloramphenicol and heptakis (2,6-di-*O*-methyl)-β-cyclodextrin: Phase solubility and spectroscopic methods. *Spectrochimica Acta, A: Molecular and Biomolecular Spectroscopy*, *83*, 570–574.

Sweetman, S. C. (2009). *Martindale: The complete drug reference* (36 ed.). London: Pharmaceutical Press.

Taupitz, T., Dressman, J. B., Buchanan, C. M., & Klein, S. (2013). Cyclodextrin-water soluble polymer ternary complexes enhance the solubility and dissolution behaviour of poorly soluble drugs, case example: Itraconazole. *European Journal of Pharmaceutics and Biopharmaceutics*, *83*, 378–387.

Wang, D., Li, H., Gu, J., Guo, T., Yang, S., Guo, Z., et al. (2013). Ternary system of dihydroartemisinin with hydroxypropyl-β-cyclodextrin and lecithin: Simultaneous enhancement of drug solubility and stability in aqueous solutions. *Journal of Pharmaceutical and Biomedical Analysis*, *83*, 141–148.

Walker, S., Diaper, C. J., Bowman, R., Sweeney, G., Seal, D. V., & Kirkness, C. M. (1998). Lack of evidence for systemic toxicity following topical chloramphenicol use. *Eye*, *12*, 875–879.

Wiest, D. B., Cochran, J. B., & Tecklenburg, F. W. (2012). Chloramphenicol toxicity revisited: A 12-year-old patient with a brain abscess. *The Journal of Pediatric Pharmacology and Therapeutics*, *17*, 182–188.

Wong, C. H., Liu, T. Z., Chye, S. M., Lu, F. J., Liu, Y. C., Lin, Z. C., et al. (2006). Sevoflurane-induced oxidative stress and cellular injury in human peripheral polymorphonuclear neutrophils. *Food and Chemical Toxicology*, *44*, 1399–1407.

Zuorro, A., Fidaleo, M., & Lavecchia, R. (2010). Solubility enhancement and antibacterial activity of chloramphenicol included in modified β-cyclodextrins. *Bulletin of the Korean Chemical Society*, *31*, 3460–3462.