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Enhanced inhibition of bacterial biofilm formation and reduced leukocyte toxicity by chloramphenicol:β-cyclodextrin:*N*-acetylcysteine complex

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ABSTRACT

The purpose of this study was to improve the physicochemical and biological properties of chloramphenicol (CP) by multicomponent complexation with β-cyclodextrin (β-CD) and *N*-acetylcysteine (NAC). The present work describes the ability of solid multicomponent complex (MC) to decrease biomass and cellular activity of *Staphylococcus* by crystal violet and XTT assay, and leukocyte toxicity, measuring the increase of reactive oxygen species by chemiluminescence, and using 123-dihydrorhodamine. In addition, MC was prepared by the freeze-drying or physical mixture methods, and then characterized by scanning electron microscopy and powder X-ray diffraction. Nuclear magnetic resonance and phase solubility studies provided information at the molecular level on the structure of the MC and its association binding constants, respectively. The results obtained allowed us to conclude that MC formation is an effective pharmaceutical strategy that can reduce CP toxicity against leukocytes, while enhancing its solubility and antibiofilm activity.

1. Introduction

Chloramphenicol (CP) (Fig. 1A) is an antibiotic with a broad spectrum of biological activity. It is used for the treatment of typhoid, salmonellosis, brucellosis, meningitis, chlamydiosis, eye infections, septic wounds, and other illnesses (Brook, 2009). It is utilized both individually and within complex formulations. However, CP may cause toxic effects on the blood production system, confused mental states, hallucinations, allergic reactions, changes in sight and hearing, initiate psychomotor disorders, and suppress intestinal microflora. The most serious adverse effect associated with CP treatment is bone marrow suppression, which is a direct toxic effect of the drug and is usually reversible, and aplastic anemia, which is idiosyncratic (rare, unpredictable, and unrelated to dose) and generally fatal. Furthermore, CP is slightly soluble in water and has a bitter taste (Shi & Zhou, 2011). These side effects and unfavourable physicochemical properties of CP may be reduced by means of its molecular complexation. The obtainment of inclusion complexes with cyclodextrins (CD) is a strategy that has been widely used due to their ability to alter the physical, chemical, toxicological, and biological properties of guest drugs, such as solubility, stability, and bioavailability (Aloisio, Longhi, & De Oliveira, 2015; Chieretin et al., 2015; Jaiswal, Bhattacharya, Mc Hale, & Duffy, 2015; Sangpheak et al., 2015). The three most common naturally occurring CDs are α-cyclodextrin (αCD), β-CD and γ-cyclodextrin (γCD), which are composed of six, seven, and eight units of D-glucopyranose linked by α-1,4 bond into a macrocycle, respectively (Connors, 1997). β-CD (Fig. 1B) was chosen for the present study

Abbreviations: CP, chloramphenicol; NAC, *N*-acetylcysteine; β-CD, β-cyclodextrin; CTC, chloramphenicol ternary complexes; FD, freeze-drying; PM, physical mixture; FT-IR, fourier transform infrared spectroscopy; SEM, scanning electron microscopy; DSC, differential scanning calorimetry; TG, thermogravimetric analysi; PXRD, Powder X-ray diffraction; ROS, reactive oxygen species; CL, chemiluminescence; MIC, Minimum inhibitory concentration; MRCNS, Methicillin-resistant coagulase negative *Staphylococcus*; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensible *S. aureus*; CV, crystal violet; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide; PMS, phenazine methosulfate.

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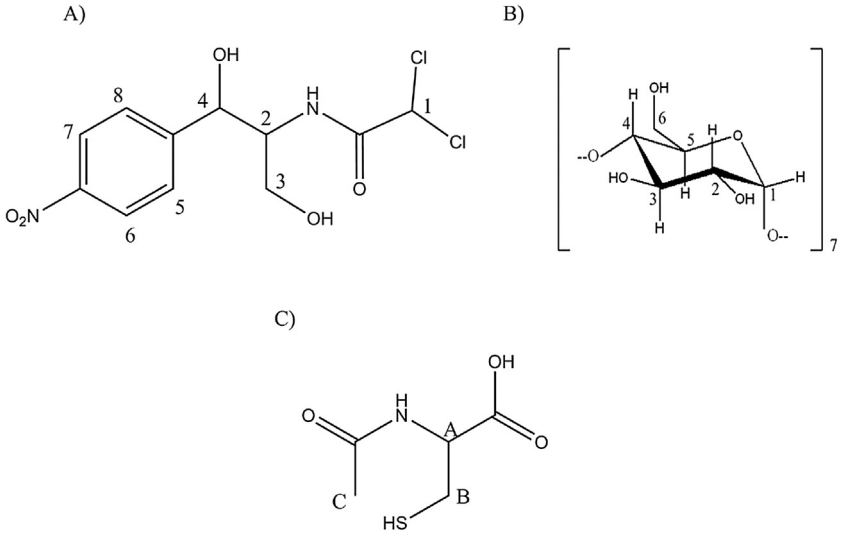

Fig. 1. Chemical structure and proton atom numbering scheme of: (A) chloramphenicol, (B) β-cyclodextrin and (C) *N*-acetylcysteine.

because it is the most common pharmaceutical excipient applied to enhance the solubility of drugs. Recently, several strategies using multicomponent complexes have been used to extend the inclusion capacity of CD or to improve the enhancement of aqueous solubility of guest drugs (Barbosa et al., 2014; García Méndez et al., 2016; Zhang, Zhang, Zhong, Chen, & Li, 2007). There are previous reports of the capability of CD to form inclusion complexes with CP. For example, Hirayama, Usami, Kimura, and Uekama (1997) studied the crystallization and polymorphic transition behaviour of chloramphenicol palmitate in 2-hydroxypropyl-β-cyclodextrin matrix. Other authors described the interaction of CP with β-CD and modified β-CD (Fatiha, Leila, Eddine, & Leila, 2013; Li, Luo & Liu, 2005; Mashhood Ali, Asmat, & Maheshwari, 2004; Ramos et al., 2013; Shi & Zhou, 2011; Zuorro, Fidaleo, & Lavecchia, 2010). Furthermore, we have previously reported the preparation and characterization of CP multicomponent complexes with β-CD and glycine or cysteine, demonstrating that these systems solve some problematic behaviours of the drug (Aiassa, Zoppi, Albesa, & Longhi, 2015).

Basically, these supramolecular systems are composed of three different molecular entities involving drug, CD, and a third component, which is selected with the aim of improving desired physicochemical, chemical or toxicological properties of a specific drug. The molecular entities selected for this purpose was *N*-acetylcysteine (NAC) (Fig. 1C), which is generally used for the medical treatment of chronic bronchitis, cancer, and paracetamol intoxication (Olofsson, Hermansson, & Elwing, 2003). NAC is an acetyl derivative of cysteine that increases tissue levels of GSH and has been widely investigated as a protective and antioxidant agent (Sahin & Alatas, 2013). Moreover, the prevention of biofilm formation and adherence to biomaterial devices is another possible role of NAC (Pérez-Giraldo et al., 1997). Biofilms are highly organized bacterial communities with functional heterogeneity that are frequently formed on biotic and abiotic surfaces. They protect bacteria from the harsh external environment via self produced matrices of extracellular polymeric substances (Kolari, 2003). Furthermore, they are also more resistant to antimicrobial agents than the same bacteria growing in a free swimming (planktonic) state (Costerton, Stewart, & Greenberg, 1999).

Continuing with our research, we studied the effects of multicomponent complexation of CP with β-CD and NAC on the aqueous solubility of drugs, antimicrobial and antibiofilm activity, and oxidative stress production in human leukocytes. CP multicomponent complex (MC) was prepared in solution, with the effects of complexation on drug solubility, affinity constant (K_C), stoichiometry, and the inclusion mode being determined by means of phase-solubility studies and ^{1}H NMR and 2D NMR spectroscopy. In the solid state, MC was prepared by the freeze-drying or simple physical mixture methods and then characterized by scanning electron microscopy and powder X-ray diffraction. The antimicrobial activity of inclusion complexes was investigated by the agar diffusion methods against the Gram-positive specie *Staphylococcus aureus* and the Gram-negative specie *Escherichia coli*. The ability of MC to inhibit biofilm growth was evaluated by crystal violet and XTT assay on *Staphylococcus* biofilms. Finally, the potential toxic effects on human leukocytes were studied using assays of reactive oxygen species (ROS) determination by chemiluminescence and fluorescence with 123-dihydrorhodamine.

Hypothesis. The combination of CP with β-CD and NAC allows enhancing the performance of the drug due to the increase in solubility because of its complexation with CD, higher activity against biofilms, and lower toxicity in presence of NAC.

2. Materials and methods

2.1. Chemicals and reagents

CP base was obtained from Parafarm (Argentina). β-CD (MW = 1135) was kindly supplied by Roquette (France). NAC, Ficoll-Hypaque (Histopaque-1077), dextran (average MW = 78.000), luminol (4-amino-2,3-dihydro-1,4-phthalazine-dione), XTT ({2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide}), DHR-123 (dihydrorhodamine-123), phenazine methosulfate (PMS), glucose, and the D_2O 99.9 atom% D 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

The phase-solubility studies for CP were performed in Milli-Q Water according to the method reported by Higuchi and Connors (1965). Excess amount of CP (100 mg) was weighed and poured into stoppered glass tubes containing a fixed concentration of NAC (10 mM) and increasing concentrations (0–13.2 mM) of β-CD. The solutions were placed in a 298 (±0.1) K thermostatized water bath

(Circulators HAAKE F3-K, Germany) for 72 h, and the resulting suspensions were vortexed for 15 s twice a day to achieve equilibrium. Specified volumes of samples were withdrawn, filtered through a membrane filter (0.45 μm), and appropriately diluted for quantitative analysis of CP in the ternary 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 used was acetonitrile with a pH 3.0 potassium phosphate buffer (10 mM) at a 50:50 mixture and a flow rate of 1.0 ml/min. Assays were performed at 298 K 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 Eq. (1):

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

The stability of the drug was determined in water at 298 K, with no drug degradation being found after 72 h of incubation.

2.3. NMR studies

The NMR spectra of pure components and MC 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 internal reference. Induced changes in the ^1H chemical shifts for CP, β-CD, and NAC ($\Delta\delta$) originated due to their complexation were calculated using the following equation:

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

The 2D-ROESY spectrum of the ternary system was 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 the solid complex CP: β-CD:NAC was carried out using the freeze-dry method (Funk, Schwabe, & Fromming, 1994). Physical mixtures were prepared by uniformly mixing the CP, β-CD, and NAC powders in a mortar.

2.5. Powder X-ray diffraction studies

X-ray diffraction (XRD) analysis was carried out for pure components and MC systems using a Philips X'Pert PRO PAN analytical powder diffractometer (Philips®, The Netherlands) with a 2θ range of 2° and 40°, a step size of 0.02°, and a scan step time of 2 s.

2.6. Scanning electron microscopy studies (SEM)

The 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 their conductivity, 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.

2.7. Microbiological studies

2.7.1. Antimicrobial activity assay

Antimicrobial susceptibility tests were performed by the agar diffusion method against American Type Culture Collection (ATCC) bacterial cells of *Staphylococcus aureus* ATCC® 25923 or *Escherichia coli* ATCC® 25922, according to Aiassa et al. (2015).

2.7.2. Minimum inhibitory concentration (MIC) determination

MIC was determined by using the standard tube dilution method as outlined by the Clinical Laboratory Standards Institute (CLSI, 2012). Strains from cultures of 24 h of methicillin-resistant coagulase-negative *Staphylococcus* (MRCNS), methicillin-resistant *S. aureus* (MRSA), and methicillin-sensible *S. aureus* (MSSA) of clinical isolates in Mueller Hinton agar (MHA) were diluted to 10^6 colony forming units per milliliter (CFU ml^{-1}), incubated for 10 min at 335 K, and then CP (0.125–512 μg/ml) or NAC (0.5–32 mg/ml) was added to them. Bacteria development was observed at 24 h of incubation, according to CLSI indications. The MIC was defined as the lowest concentration of drug that inhibited visible growth after 24 h of incubation at 335 K.

2.7.3. Antibiofilm activity assay

2.7.3.1. Biofilm formation and treatment. Biofilms were formed in 96 well tissue culture plates containing 200 μl of MRCNS, MSSA or MRSA cell suspension (1×10^6 cells ml^{-1}) in trypticase soy broth (TSB) supplemented with 0.25% glucose per well to promote biofilm formation. Plates were incubated for 24 h at 335 K on an orbital shaker (130 rpm). Finally, planktonic cells were removed carefully, and the biofilm was washed twice with 200 μl of 0.9% NaCl. The biofilms were incubated in fresh nutrient medium containing CP (8 μg ml^{-1} = MIC and 10x MIC), NAC (4 mg ml^{-1} = MIC and 10 × MIC), and a combination of both in complexation with β-CD. XTT and crystal violet (CV) assays were performed after 24 h of exposure to antimicrobial agents (alone and in combination).

2.7.3.1.1. XTT assay. The quantification of biofilm cellular activity was assessed through the XTT reduction assay. After exposure to CP, NAC or MC, the biofilms were washed with 0.9% NaCl. Then, 250 μl of a solution containing 200 mg l^{-1} of XTT and 20 mg l^{-1} of PMS was added to each well. The microtiter plates were incubated for 3 h at 335 K in darkness. The absorbance was measured at 490 nm in a Biotek Synergy 2 multimode microplate reader (Leite et al., 2013).

2.7.3.1.2. Crystal violet assay. CV was used as indicator of total biofilm biomass. For the measurement of this parameter, biofilms were washed with 250 μl of 0.9% NaCl, and then 250 μl of methanol was added and left to act for 15 min. Afterwards, methanol was removed, and 250 μl of crystal violet 1% (v/v) was added (5 min). The wells were washed with distilled water, and finally alcohol:acetone 30:70% (v/v) was added to them. The absorbance was measured at 595 nm. Controls were cells not exposed to CP (positive control) and cells exposed either to CP or to pure NAC. All experiments were carried out in triplicate and repeated three times (Leite et al., 2013).

2.8. Reactive oxygen species determination

For ROS analysis, peripheral blood samples with heparin were obtained during the same morning. Leukocytes were isolated by a combined dextran/Ficoll-Hypaque 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} leukocyte for the assays of chemiluminescence and fluorescence with DHR-123.

2.9. 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 leukocyte CL was measured in the presence of luminol. 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 β-CD (0.3 mM), 3) 0.2 ml of CP:β-CD (0.3 mM of CP and 0.3 mM of β-CD), and 4) 0.2 ml of CP:β-CD:NAC (0.3 mM of CP, 0.3 mM of β-CD, and 10 mM of NAC). 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).

2.10. Fluorescence assay with DHR-123

Leukocyte ROS was also determined by the formation of the fluorescent compound rhodamine-123 from DHR-123 with some modifications according to Nemzer et al. (2014). 60 μl samples of leukocyte suspensions were incubated with 20 μl DHR solution (10 μl M), 60 μl buffer, and 60 μl of 1) CP (0.3 mM) or 2) CP:β-CD:NAC (0.3 mM of CP, 0.3 mM of β-CD, and 10 mM of NAC). A Biotek Synergy 2 multimode microplate reader was used to record the intensity of the fluorescent compound rhodamine-123. The fluorescence emission was recorded at 535 nm (20 nm) and upon excitation at 485 nm (20 nm). Results were expressed in relative fluorescence units (RFU) *versus* time.

2.11. Statistical analysis

Data from 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. Characterization of CP:β-CD:NAC complex

3.1.1. Phase solubility analysis

The phase solubility diagram of CP with β-CD and NAC (data not shown) obtained by plotting the changes in guest solubility as a function of β-CD concentration can be classified as an A_L type according to Higuchi and Connors (1965). It showed a linear increase in drug solubility, which is indicative of the formation of soluble complexes. The corresponding stability constant value calculated from the phase-solubility diagram was 75 ± 3 M^{-1} with an increase in drug solubility of 1.5 fold. This K_C value was less than that corresponding to the binary CP:β-CD complex (180 ± 12 M^{-1}) that we reported previously (Aiassa et al., 2015). This behaviour may be due to a competitive effect between CP and NAC to establish interactions with β-CD.

3.1.2. NMR spectroscopic studies

^{1}H NMR experiments were carried out in order to study the interaction and inclusion modes of CP in MC. Comparisons were made between pure components and their corresponding signals in MC. As seen in Table 1, the presence of CP and NAC induced major variations in the chemical shifts of the β-CD protons located inside the cavity (H_3 and H_5), while only minor changes were observed for protons located outside it (H_1, H_2 and H_4). The shielding effect observed for H_3 and H_5 protons can be attributed to the insertion of the CP aromatic ring into the β-CD cavity. In the case of CP, a deshielding effect was observed on all protons, with the exception of H_3 that exhibited a shielding effect. The downfield displacement observed for H_1, H_2 and H_4 CP protons can be due to the hydrogen bond interactions of the drug with β-CD and/or NAC, while the displacement of the drug aromatic protons can be explained by their inclusions inside the β-CD cavity. Finally, a deshielding effect was observed for H_A (0.0121 ppm) and H_B (0.0044 ppm) protons of NAC. This effect might be explained by the presence of hydrogen bond interactions between the carbonyl and amine groups of NAC with β-CD hydroxyl groups or carbonyl groups of CP.

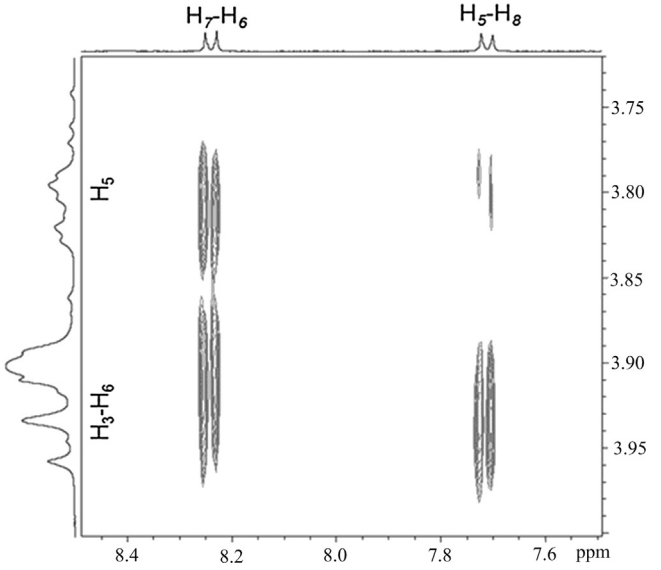

Fig. 2. Partial contour plot of the 2D ROESY spectrum of CP:β-CD:NAC system.

The inclusion of CP molecule in the macromolecule cavity was confirmed by the 2D ROESY techniques. The 2D NMR spectrum (Fig. 2) showed several intermolecular cross peaks between H_3 and H_5 protons of β-CD and H_5–H_8 and H_7–H_6 protons of CP, demonstrating that the aromatic ring of the drug was deeply inserted into the CD cavity.

When we compared the results of these studies with a previous report (Mashhood Ali et al., 2004), we concluded that NAC interacts with the inclusion complex, but without substantially modifying the CP insertion mode. This behaviour is similar to that previously found for multicomponent complexes prepared with Gly or Cys (Aiassa et al., 2015).

3.1.3. Scanning electron microscopy (SEM) and powder X-ray diffraction (XRD) studies

The shape and structure of CP:β-CD:NAC complex particles prepared by the freeze-dried method (FD) were assessed by SEM (Fig. 3) and XRD (Fig. 4), respectively, and were compared with the pure materials and CP:β-CD:NAC system prepared by physical mixture (PM). In SEM images of CP:β-CD:NAC FD, it was observed that this product had a significantly different morphology with respect to the pure materials and the PM with small size particles and a tendency to aggregation. The XRD patterns showed that the CP, β-CD, pure NAC, and the PM system were crystalline solids, while the CP:β-CD:NAC FD was an amorphous product.

These results indicate that the CP:β-CD:NAC system prepared by the FD method is a new solid phase of amorphous nature which differs from the system obtained by the PM where the components do not interact.

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

System	CP Protons	Δδ (ppm)	NAC protons	Δδ (ppm)	βCD protons	Δδ (ppm)
CP:βCD	H1	−0.0361			H1	−0.0508
	H2	0.1023			H2	−0.0366
	H3	Overlapped			H3	−0.1068
	H4	0.0962			H4	−0.0419
	H5–H8	0.0891			H5	−0.18395
	H7–H6	−0.0489			H6	−0.0429
CP:βCD:NAC	H1	0.0204	HA	0.0121	H1	−0.0121
	H2	0.0919	HB	0.0044	H2	−0.0020
	H3	− 0.0332	HC	−0.0002	H3	−0.0607
	H4	0.0885			H4	−0.0060
	H5–H8	0.0890			H5	−0.0982
	H7–H6	0.0065			H6	−0.0051

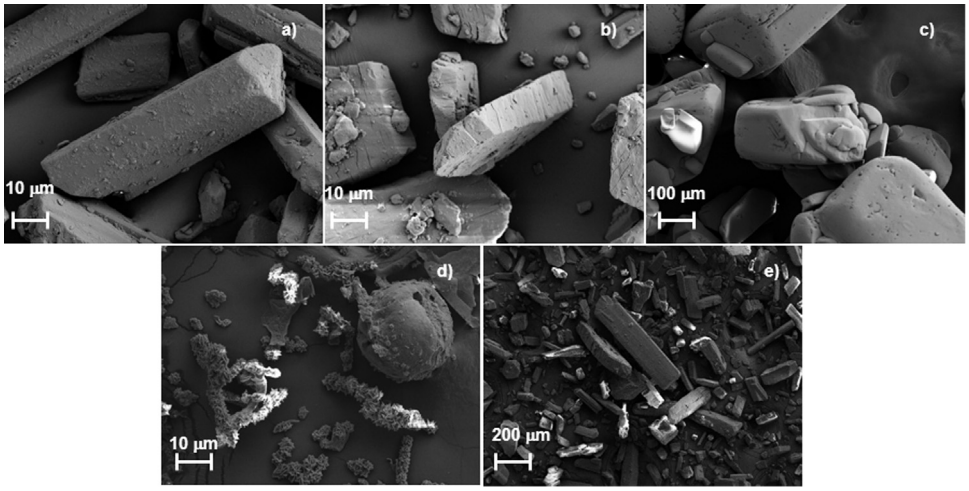

Fig. 3. Scanning electron microphotographs of: (a) CP, (b) β-CD, (c) NAC, (d) CP:β-CD:NAC PM system and (e) CP:β-CD:NAC FD system.

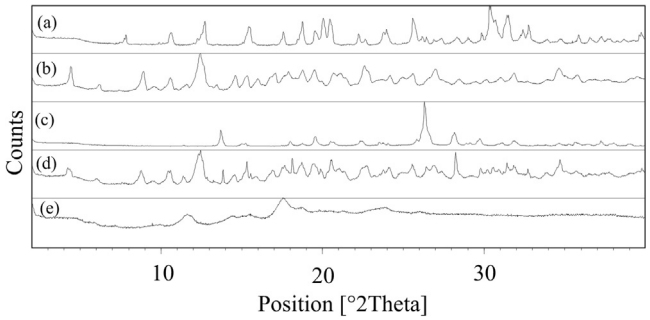

Fig. 4. X-ray diffractograms of: (a) CP, (b) β-CD, (c) NAC, (d) CP:β-CD:NAC PM system and (e) CP:β-CD:NAC FD system.

3.2. Microbiological studies

3.2.1. Antimicrobial activity

The antimicrobial activity of CP and its binary and ternary systems with β-CD and NAC against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) species were determined by the agar diffusion methods with the purpose of assessing the ability of β-CD 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 activity. CP binary and ternary systems did not show significant differences against *S. aureus* and *E. coli* with respect to the pure CP inhibition zones which were 24.5 ± 0.7 mm, 25 ± 1 mm, and 24.5 ± 0.7 mm for CP, CP:β-CD and CP: β-CD:NAC in *S. aureus*, respectively, 25 ± 1 mm for CP and CP:β-CD, and 25.5 ± 0.5 CP: β-CD:NAC in *E. coli*. These results showed that CP complexation did not interfere with the microbiological activity of the drug, which allowed us to infer that the interactions between the components in the complex were sufficient to improve the solubility of the drug, but not strong enough to reduce its microbiological potency.

3.2.2. Antibiofilm activity

The MIC values of CP and NAC for the three strains of *Staphylococcus* (MRCNS, MRSA, and MSSA for planktonic cells) determined in this study were of 8 μg ml^{-1} for CP and 4 mg ml^{-1} for NAC.

Fig. 5A presents the results expressing the decrease in the metabolic activity measured by the XTT reduction assay after treatment with CP, pure NAC, and MC with β-CD. These results showed that the MC [CP (MIC) + NAC (10 × MIC) + β-CD 13.2 mM] caused a very significant reduction in cellular metabolic activity.

The variation in total biofilm biomass assessed by CV staining also confirmed the effect of the antimicrobial agents tested. Fig. 5B shows that although the reduction in the metabolic activity was more remarkable than in the biomass of *Staphylococcus* strains, CP (MIC) + NAC (10 × MIC) + β-CD 13.2 mM caused the biggest reduction in biofilm biomass.

Overall, a general promotion of biofilm weakness may be a potential tool for the human immune system to fight *Staphylococcus* biofilm associated infections. In this work, the MC of CP:β-CD:NAC demonstrated a synergistic effect; therefore, it is more effective than CP (10 × MIC) and has a similar effect to that of pure NAC (10 × MIC).This combination is able to reduce the metabolic activity in biofilm populations of *Staphylococcus*. Previous studies have shown that this detachment and dispersive action of NAC in biofilms makes the biofilm-associated bacteria more suscepti-

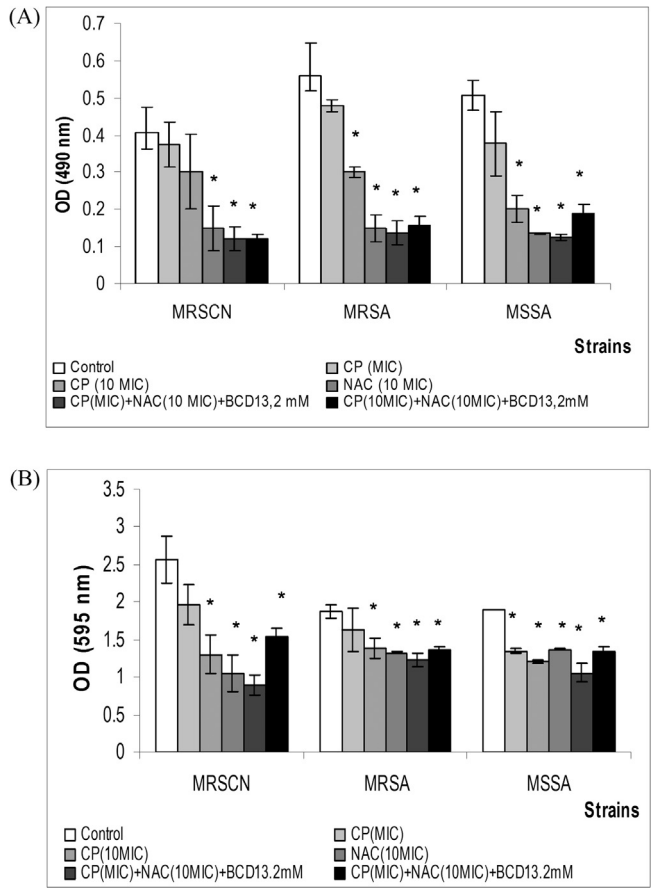

Fig. 5. Effect of CP, NAC and MC on: (A) metabolic activity determined by XTT assay or (B) biofilm biomass quantified by CV assay of *Staphylococcus* strains. Error bars represent standard deviation. *p < 0.05 with respect to the control.

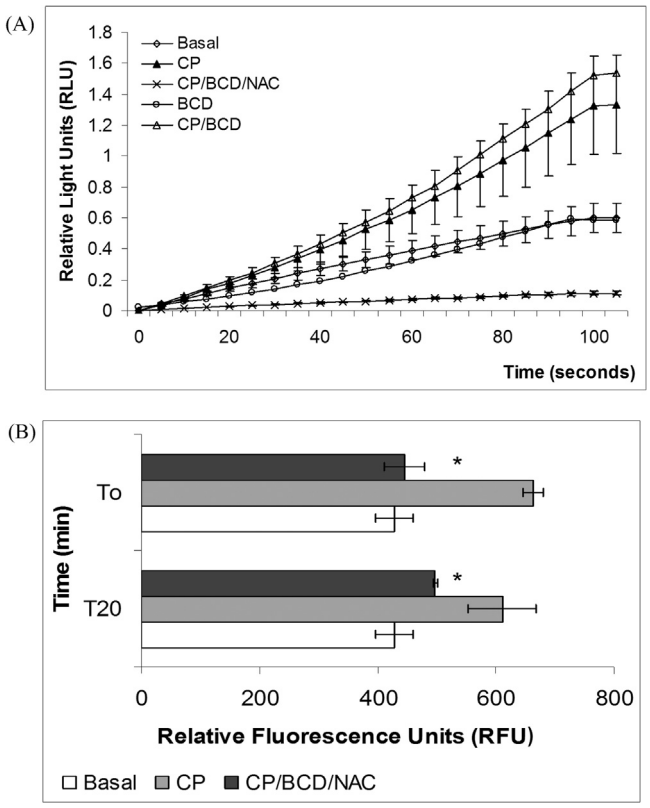

Fig. 6. Reactive oxygen species induced by CP quantification of human leukocytes by (A) Chemiluminescence assay, (B) Fluorescence assay with DHR-123. *p < 0.05 with respect to pure CP.

ble to other antimicrobial agents (Aslam, Trautner, Ramanathan, & Darouiche, 2007) (as in the present association with CP). In this case, pure NAC at a concentration of 40 mg ml^{-1} also presented a very high inhibitory effect on planktonic cells and markedly reduced the metabolic activity in biofilms. Moreover, NAC is widely used in medical practice via inhalation and oral and intravenous routes and has an excellent safety profile (Kao et al., 2003), making it a suitable third component to be used in a formulation.

3.3. ROS determination

Free radical reactions have been suggested to be involved in the toxic effects of several antibiotics (Cuffini et al., 2002; Halliwell & Gutteridge, 1989; Okuyan et al., 2005; Tullio et al., 2004; Vazifeh, Abdelghaffar, & Labro, 2002). Generally, the cytotoxic effects of therapeutic drugs include diverse metabolic changes that affect the normal functioning of host cells, with oxidative stress being one of the alterations provoked. Similarly, some antibiotics seem to affect the oxidative state of cellular components, for example the action of CP on cytochrome P450. This is related to enzymatic oxidation via an increase in ROS, while the coadministration of antioxidant vitamins may attenuate its toxic action (Farombi, Adaramoye, & Emerole, 2002). Our previous investigation has demonstrated that the antioxidants, glycine and cysteine, reduce the oxidative stress mediated by ROS in human leukocytes (Aiassa et al., 2015). Therefore, in this work the effect of NAC on this MC was evaluated through the toxicity mediated by ROS in leukocytes since NAC is a potent antioxidant and may function directly as a ROS scavenger or indirectly as a cysteine source or a glutathione precursor (Atkuri, Mantovani, & Herzenberg, 2007). Our results (Fig. 6A) confirmed the presence of oxidative stress in leukocytes produced by CP since ROS production measured by CL was significantly greater in leukocytes treated with CP (100 μg/ml) than in the untreated ones ($p < 0.05$). Pure β-CD did not produce an increase in ROS, while the CP: β-CD complex behaved as pure CP and showed a rise in ROS. Finally, when the MC was evaluated, the production of ROS was significantly lower than that of the untreated samples, which suggests that NAC can act as a potent ROS inhibitor/scavenger. Therefore, this reduction shows the capability of this MC containing NAC to reduce the toxic effects of CP without affecting its microbiological activity. The effectiveness of NAC in reducing the toxicity of CP mediated by ROS can be reaffirmed by the results obtained by ROS determination by means of the formation of the fluorescent compound rhodamine-123 from DHR-123 (Fig. 6B).

4. Conclusions

This study has demonstrated that when NAC is used as a third component, it can produce a protective effect on the increase of ROS induced by CP in leukocytes. Moreover, the solubility of CP and the CP activity against *Staphylococcus* biofilms can be improved by inclusion complexation with β-CD and NAC, with the complexation of this antimicrobial drug not interfering with its microbiological activity. According to the CP:β-CD:NAC complex behaviour, it can be concluded that they are potentially useful applications for the future formulation of dosage forms of chemotherapeutic drugs that produce toxicity mediated by ROS.

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