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ORIGINAL ARTICLE

Ocular Application of Dirithromycin Incorporated Polymeric Nanoparticles: an *In Vitro* Evaluation

Diritromisin Yüklü Polimerik Nanopartiküllerin Oküler Uygulanması: *İn Vitro* Değerlendirme

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ABSTRACT

Objectives: Ocular drug delivery is a difficult challenge especially with topical intillation which results in rapid drainage and non-productive drug absorption. For the improvement of the pre-corneal retention time and enhancing the corneal permeability, colloidal drug delivery systems play an important role in enhancement of the ocular bioavailability. In this study, dirithromycin incorporated Kollidon® SR-based polymeric nanoparticles, an antibacterial agent, were formulated for the efficient treatment of severe ocular bacterial infections.

Materials and Methods: In this study, dirithromycin was incorporated into the Kollidon® SR-based nanoparticles by spray drying method. *In vitro* characteristic properties were evaluated in detail during the storage period of three months at three different conditions.

Results: The results of *in vitro* analyses revealed that characteristic properties of the particles were remained unchanged during the storage period of three months.

Conclusion: Kollidon® SR-based polymeric nanoparticles are good candidates for drug delivery systems in the treatment of severe ocular bacterial infections with dirithromycin.

Key words: Dirithromycin, Kollidon® SR, polymeric nanoparticles, ocular drug delivery

ÖZ

Amaç: Özellikle gözyaşı üretimi ve kırpma refleksleri gibi gözün koruyucu mekanizmalarına bağlı olarak göze topik olarak uygulanan formülasyonların göz yüzeyinden hızla uzaklaştırılması ve korneal yüzeyden verimsiz absorbsiyonu söz konusu olmaktadır. Prekorneal tutunma süresinin ve korneal permeabilitenin arttırılması ile oküler biyoyararlanımın arttırılmasında kolloidal ilaç taşıyıcı sistemler büyük önem taşımaktadır. Bu çalışmada antibakteriyel etkili bir madde olan diritromisin yüklü Kollidon® SR yapılı polimerik nanopartiküller şiddetli oküler bakteriyel enfeksiyonların etkin tedavisi amacı ile hazırlanmıştır.

Gereç ve Yöntemler: Bu çalışmada diritromisin Kollidon® SR yapılı polimerik nanopartiküllere püskürterek kurutma yöntemi ile yüklenmiştir. Nanopartiküllerin *in vitro* karakteristik özellikleri üç farklı ortamda üç ay süresince detaylı olarak incelenmiştir.

Bulgular: *İn vitro* analiz sonuçları parçacıkların üç aylık saklama süresince karakteristik özelliklerini koruduklarını göstermiştir.

Sonuç: Kollidon® SR yapılı polimerik nanopartiküllerin şiddetli oküler bakteriyel enfeksiyonların diritromisin ile tedavisinde oldukça etkili ilaç taşıyıcı sistem adayları olduklarını göstermiştir.

Anahtar kelimeler: Diritromisin, Kollidon® SR, polimerik nanopartiküller, oküler ilaç taşıyıcı sistemler

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INTRODUCTION

The topical instillation of the ocular formulations is the primary choice application method for ocular therapy.[1] However, the bioavailability of ophthalmic drugs via topical route result in a short duration at the therapeutic concentration, due to the several protective mechanisms of the eye like lacrimal secretion, high tear turnover and blinking reflex.[2,3] To overcome the poor bioavailability of drugs (5-10% of the applied dose) from ocular dosage forms after topical application, researchers mostly developed mucoadhesive colloidal drug delivery systems for extended corneal/conjuctival contact time.[4-7]

Most cases of acute infections, topical antibacterial treatment offers several benefits like shorter disease duration; prevention of spread of infection; reduction in side effects, and reduced disease recurrence.[8] In the eye bacterial, fungal and viral pathogens can produce severe disorders like conjunctivitis, keratitis, blepharitis, corneal ulcers and are one of the causes of ophthalmia neonatorum for the infants which may lead to the loss of an eye when being untreated sufficiently.[9,10]

Bacteria and other microorganisms are far more resistant to adverse situations than animal cells and can withstand environments that would be quickly lethal to us in many cases.[9]

Macrolides are one of the antibacterial agent group that are being used for the treatment of proposed disorders. All of the macrolides have a similar mechanism of action in that they selectively bind to the 50S subunit of the bacterial ribosome while not binding to the mammalian 80S ribosomal subunit, which accounts, in part, for their safety and selectivity of action.[11]

Erythromycin is the first member of the macrolide group and many derivatives were synthesized afterwards.[12] Dirithromycin is the second generation of semisynthetic macrolide derived from erythromycin with a 14-membered lactone ring.[13] It is acid stable and better absorbed with higher bioavailability than for erythromycin however its oral bioavailability results in between 6-14%.[11,14]

Furthermore, in addition to their antibacterial activities, macrolides such as azithromycin exhibit potent anti-inflammatory activities.[10] Despite being well tolerated, macrolides have a number of important side effects such as gastrointestinal adverse reactions QT interval prolongation, hepatotoxicity, ototoxicity, color vision loss and interactions with other drugs because of inhibition of drug metabolism.[15,16] For the enhancement of low bioavailability data and requirement of safer formulations dirithromycin was incorporated into polymeric nanoparticles in this study.

Kollidon® SR is polyvinyl acetate [polyvinyl alcohol (PVA), 80%] and [polyviniyl pyyrolidone (PVP), 20%] based mixture mostly used for pH-independent sustained release matrix tablets.[17-19] When PVA part gives to a tablet/particle structural integrity, water soluble PVP part leaches out forming pores where the active agent diffuses out.[17] Kollidon® SR has demonstrated to have no acute toxicity and to be not irritating to the skin or mucous membranes.[20] Kollidon® SR contains no ionic groups therefore its sustained release properties are unaffected by ions or salts.[21]

Considering the safety and the modification opportunities of the polymer novel application route for Kollidon® SR was studied for the first time in this study for efficient treatment of ocular bacterial infections using dirithromycin.

MATERIALS AND METHODS

Materials

Dirithromycin and Kollidon® SR was kindly gifted by Abdi İbrahim (İstanbul, Turkey) and by BASF (İstanbul, Turkey) respectively. Methanol was purchased from Merck (Darmstadt, Germany). *Staphylococcus aures* [American Type Culture Collection (ATCC 25923)] and *Pseudomonas aeruginosa* (ATCC 27853) strains were obtained from ATCC. All other reagents used were of analytical grade.

Methods

Preparation of nanoparticles

Spray-drying method was used for the preparation of nanoparticles.[22-24] Briefly, accurately weighed Kollidon® SR (1 g) was dissolved in methanol (60 mL). Dirithromycin was added to the mixture under mild agitation (150 rpm). Final transparent solution was then spray-dried using a Nano Spray Dryer (B-90, BUCHI Labortechnik AG, Flawil, Switzerland) with an inlet temperature of 90˚C±1˚C (outlet temperature of 35˚C±5˚C). White dry powders were collected and kept in tightly-closed and coloured vials at room temperature until being analyzed.

Placebo nanoparticles were prepared as described above without the addition of active agent.

Characterization of nanoparticles

Morphology

The microstructural characterization of the nanoparticles prepared were investigated using scanning electron microscope (SEM) (Carl Zeiss SUPRA 50 VP, Oberkochen, Germany) at 25˚C±2˚C.

Particle size and zeta potential

Particle size, polydispersity index (PDI) and zeta potential measurements were performed on freshly prepared samples using Malvern Nano ZS (Zetasizer Nano Series, Worcestershire, UK). Samples of all nanoparticles were dispersed in double-distilled water (adjusted to a constant conductivity of 50 μS/cm^2 using 0.9% NaCl) just prior to analyses.[25] All analyses were repeated in triplicate at 25˚C±2˚C.

pH value

*p*H values of the nanoparticle dispersions were analyzed at 25˚C±2˚C by WTW Profi Lab (pH 597, Weilheim, Germany). All analyses were repeated in triplicate.

Differential scanning calorimetry

Structural and crystallinity changes of dirithromycin and the Kollidon® SR due to the formulation steps were evaluated using differential scanning calorimetry (DSC) (DSC-60, Shimadzu Scientific Intruments, Columbia, MI, USA). Analyses were performed under nitrogen (flow rate of 50 mL/min) at 30-300˚C

temperature range with a constant heating rate of 10°C min^{-1}. DSC thermograms of pure materials were used as references for the comparison of the possibility of crystallinity changes of the structures.

X-ray diffractometry

For the evaluation of the crystallinity changes X-ray diffraction (XRD) analyses were performed (Rikagu Corporation D/Max-3C; Tokyo, Japan) within the range of 2-40° at 2θ with 2°/min scanning rate and using 40 kV voltage with 20 mA current intensity level. Analyses spectra of pure polymer and pure dirithromycin were used as references for evaluating the structural changes of the materials throughout formulation stages.

Fourier transform infrared spectrophotometry

IR Prestige-21 (Shimadzu, Tokyo, Japan) was used for fourier transform infrared (FTIR) analyses. Deuterated triglycine sulfate doped with L-alanine detector was used with Germanium-coated KBr plate beam splitter at 4000-500 cm^{-1} range. FTIR spectra of pure dirithromycin and polymer were used as references.

Nuclear magnetic resonance

For the evaluation of the interactions between the active agent and the polymer ^{1}H-NMR analyses were performed on UltraShield™ CPMAS NMR (Brucker, Rheinstetten, Germany) using deuterated chloroform ($CDCl_3$) as solvent. Spectra of pure dirithromycin and Kollidon® SR were used as references.

Determination of dirithromycin

A modified high-performance liquid chromatography (HPLC) method was used for the determination of dirithromycin.[26,27] Shimadzu 20 A (Tokyo, Japan) with Shimadzu Shim-Pack CLC-ODS column (Tokyo, Japan; column diameter: 4.6 mm, column length: 15.0 cm, particle diameter: 5 µm and particle size: 100 Å) was used as the instrument. Water:methanol:pbs buffer:acetonitrile (9:19:28:44, v/v/v/v, pH: 7.5) was used as the mobile phase with a flow rate of 1.0 mL/min. 20 µL constant amount of samples was injected via an Automatic Injector (Shimadzu, Tokyo, Japan) and Photodiode Array Detector (Shimadzu, Tokyo, Japan) was used at 205 nm. Column temperature was set to 30°C. Validation studies were performed for data reliability.

Drug loading and encapsulation efficiency

Accurately weighed particles (50 mg) were dissolved in 5 mL methanol while the same amount of particles were dispersed in 2-propanol (5 mL), vortexed for 1 min and centrifuged at 2000 rpm for 10 min for the determination of total dirithromycin content (DIR_T) and dirithromycin adsorbed on the nanoparticle surfaces (DIR_S), respectively. Supernatants were analyzed by HPLC after proper dilutions (n=3).

Drug loading and encapsulation efficiency (EE) were calculated according to Equation 1 and Equation 2 respectively.

$$\text{Drug loading (\%)} = [(DIR_T) / (\text{Particle weight})] \times 100$$

Equation (1)

$$\text{EE (\%)} = [(DIR_T - DIR_S) / (DIR_T)] \times 100$$

Equation (2)

In vitro release study

The dialysis bag diffusion method was used to analyze the *in vitro* drug release studies of dirithromycin incorporated Kollidon® SR nanoparticles.[28,29] *In vitro* release profiles of dirithromycin were investigated in freshly prepared simulated tear fluid (STF) at pH 7.4.[30-32] Briefly, spray-dried nanoparticles containing 5.0 mg dirithromycin were put in dialysis bags. Bags were closed tightly from both ends and were immersed in the dissolution medium containing 30 mL STF at 34°C±1°C on a water bath using continuous magnetic stirrer with a stirring rate of 150 rpm. 1 mL of samples were collectes at predetermined time intervals and dirithromycin content of the samples was analyzed using HPLC method as described at the previous section and release profile of the pure dirithromycin was used as a reference for better evaluation of the profiles. Each experiment was repeated three times.

Evaluation of the cytotoxicity

The toxicity of the nanoparticles prepared were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay.[33,34] Briefly, cells were suspended in Dulbecco's Modified Eagle's Medium solution (containing 10% fetal bovine serum) and $2x10^4$ cells/mL were seeded into 96 well plates and cultured for 24 hrs. Formulations with different concentrations were added to the suspension and the mixtures were incubated at 37°C (under 5% CO_2 and 95% air) for 24 and 48 hrs. After the incubation period, 20 µL (5 mg/mL) of MTT dye was added to each well and incubated for more 2 hrs. (at 37°C) for the transformation of MTT to formazan salt by the presence of the living cells. Formazan crystals were extracted using 200 µL dimethyl sulfoxide (DMSO) and the amount of the dye was determined spectrophotometrically at λ=540 nm with microplate reader (Victor X5, Perkin Elmer, England).

Microbiological assay

The antifunfal activity of the formulations were evaluated with M27-A2 standard Microbroth Dilution Method.[35] Briefly formulations were dissolved in DMSO within the concentration of 400 µg/mL-0.78 µg/mL and transferred to the 96 well plates with a concentration of 100 µL. *S. aures* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) were inoculated at the concentrations of $5x10^5$ CFU/mL. Positive and negative control groups were used as references. Resazurin was added to the plates up to the final concentration of 20 µg/mL after the 24 h incubation period at 36°C. The plates were analyzed with Synergy™ HT (Bio-Tek, USA) and the minimum inhibitory concentration (MIC) was evaluated as the lowest concentration that inhibits the visible microbial growth.[36]

Stability of the formulations

For the evaluation of the stability of the formulations prepared, samples were kept at different temperatures (25°C±2°C - 60%±5 RH; 40°C±2°C - 75%±5 RH, 5°C±3°C) during the storage period of 3 months and the particle size, PDI, zeta potential, pH and DSC analyses were evaluated on the day of production and at the end of 3 months for the evaluation of the changes in the characteristic properties of the nanoparticles.

RESULTS AND DISCUSSION

Compositions of the Kollidon® SR nanoparticles prepared were given in Table 1.

Duration of active agent on the ocular surface is mostly particle size dependent.[37] Drug can be absorbed into ocular tissues from precorneal pocket by small sized particles while on the other hand, longer retention times on the ocular surface and slow drug dissolution can be achieved by bigger sized particles. However condisering the irritation and tolerability issues particle sizes smaller than 10 µm are prerequisite for ocular administration.[37-39]

Particle size, zeta potential, pH and active agent contents of the Kollidon® SR based particles were summarized in Table 2.

Particle sizes of KD1 and KD2 formulations were found to be 329.6±6.8 nm and 522.2±24.4 nm, respectively, with relatively homogenous size distribution considering the PDI values (Table 2). Increased encapsulation has influenced the particle size however particle sizes remained in the nanometer range which will be convenient for topical ocular application. During the storage period of 3 months particle sizes were slightly changed (Table 2) showing that the storage conditions did not change the ocular applicability potential of the particles by remaining below the limit of 10 µm.[39]

Particle size and surface charge and are the two most frequently referred factors that are responsible for the enhanced biological effects of nanoparticles including cellular uptake, toxicity and dissolution.[40] Despite giving informations about the interactions with the ocular tissues, zeta potential values also gives preliminary data for the prediciton of the physical stability of particle dispersions.[41] Guidelines classifying nanoparticle dispersions with zeta potential values of ±0-10 mV, ±10-20 mV and ±20-30 mV and >±30 mV as highly unstable, relatively stable, moderately stable and highly stable, respectively.[40,42]

Since the corneal and conjunctival surfaces are negatively charged, main approach for the enhancement of ocular bioavailability cationic drug delivery systems are being preferred considering possesment of enhanced bioavailability due to the electrostatic interactions between the surfaces.[24,43-46] However anionic drug delivery systems also can enhance ocular bioavailability by the help of monocarboxylate transport which processes a proton or Na^+-coupled lactate systems in epithelial cells which may enhance the ocular bioavailability of the anionic drugs in a great extent.[47]

In this study zeta potentials were valued as -19.5±0.3 mV and -25.5±0.1 mV for KD1 and KD2 formulations respectively (Table 2). After 3 month storage period potentials were valued within the range of -20.1±4.1 mV and -26.5±0.3 mV indicating physical stability of the nanoparticles during the storage period (Table 2). Considering negatively charged polymeric nanoparticles prepeared, active transport might be the main mechanism of the enhanced bioavailability of dirithromycin by topical application.[47]

The pH and buffering of ocular formulations are very important since the pH changes are the main reason of the stability problems as well as the discomfort, safety and efficacy issues of the formulations prepared. Therefore ideally pH of the formulations would be buffered to pH 7.4 considering the physiological pH of the tear fluid.[48] Since the pH values of the formulations were 5.04±0.00 for placebo KD, 7.39±0.01 for KD1 and 7.90±0.00 for KD2 no adjustments were required for dirithromycin incorporated nanoparticle formulations in our study (Table 2). After 3 month storage period pH values were remained within the range of ocular tolerability.[49]

Dirithromycin was reported to crystallize into different polymorphic forms in different solvents, such as nonsolvated crystal forms (1 and 2), 1-propanol solvate, cyclohexane trisolvate and acetonitrile-trihydrate.[50,51] Therefore it is very important to evaluate morphological changes of dirithromycin and Kollidon® SR during and after formulation steps. In our study structural changes were evaluated by DSC analyses. Pure dirithromycin demonstrated a melting point at 191.3°C showing the crystalline structure (Figure 2a). No peaks were detected for both placebo formulation and pure Kollidon® SR as expected due to the amorphous structure of the polymer. DSC thermogram of the physical mixture of the dirithromycin with Kollidon® SR showed the stability of the active agent in the polymeric carrier owing to the presence of dirithromycin in crystalline structure. And the absence of the endothermic peak of dirithromycin in the formulations showing that the

Table 1. Composition of Kollidon® SR nanoparticles prepared

Code	Kollidon® SR (g)	Dirithromycin (g)	Methanol (mL)
Placebo KD	1	—	60
KD1	1	0.10	60
KD2	1	0.15	60

KD: Kollidon®, Dirithromycin

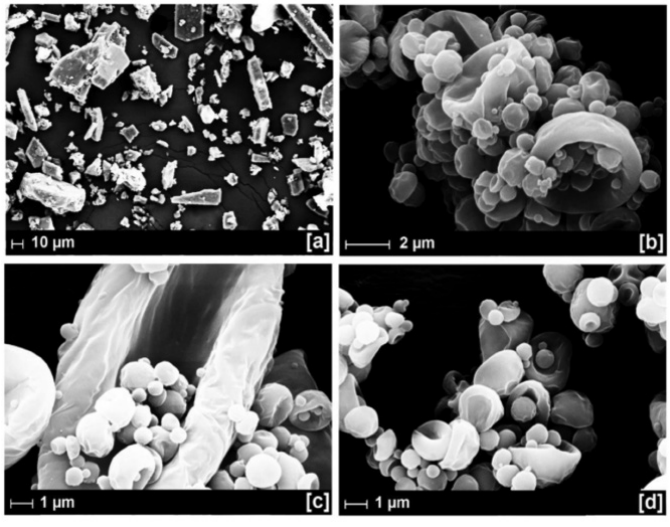

Figure 1. Scanning electron micrographs of pure dirithromycin and nanoparticles prepared, a) Dirithromycin, b) Placebo KD, c) KD1, d) KD2

KD: Kollidon®, Dirithromycin

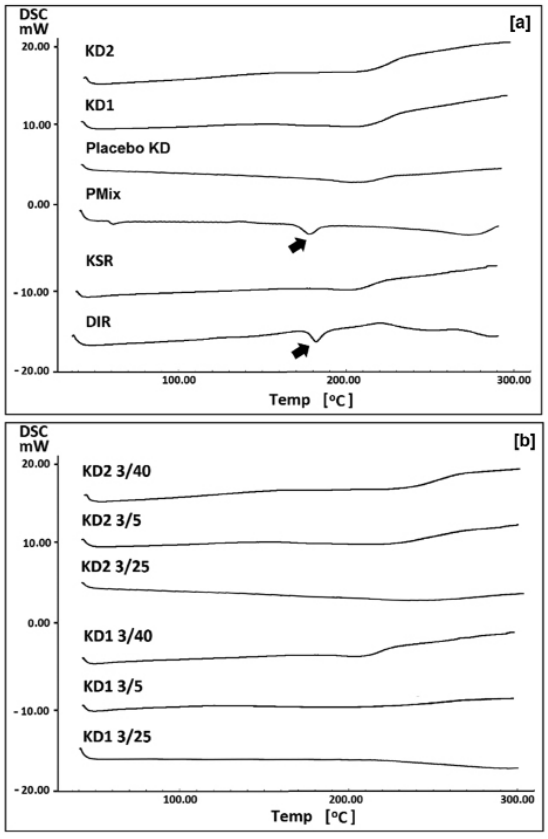

Figure 2. Differential scanning calorimetry thermograms of pure dirithromycin and the nanoparticles prepared, a) At the day of production, b) After the storage period of 3 months

DIR: Dirithromycin, KSR: pure Kollidon® SR, PMix: Physical mixture of dirithromycin with Kollidon® SR, Stability codes as x/y; x represents the storage month while y represents the storage conditions

dirithromycin was molecularly dispersed within the amorphous polymeric structure (Figure 2a). After the storage period of 3 months amorphous state of the polymeric structure remained unchanged showing that the particles were not affected from the storage conditions (Figure 2b).[52]

XRD is one of the most important characterization tools used in solid state chemistry and materials science for the determination of the size and the shape of the unit cell for polycrystals in nanoscale.[53,54] Diffraction pattern gives information of the crystalline/amorphous structure of the materials.[54] Since the polymorphic changes of the active agents are important factors which might affect the dissolution rate and the bioavailability of the applied drug, possibility of the structural changes must be monitored.[55] XRD also gives information about the size of the particles with the help of measurement of the smallest unfaulted regions or coherently scattering domains of the material.[56]

In XRD profiles dirithromycin showed sharp peaks at 2θ-scattered angles indicating crystalline structure (Figure 3). Pure Kollidon® SR showed semicrystalline structure (marked with arrows) however after spray drying process polymer transformed to the amorphous state which gives spaces for the incorporation of the active agent within the polymeric network. No signals were detected in the spectra of KD1 and KD2 (Figure 3) showing that the dirithromycin was dispersed within the amorphous polymer as indicated by DSC analyses results.

FTIR analysis method based on the selective absorption of light by the vibration modes of specific chemical bonds in the sample therefore FTIR analyses gives concise informations about the interactions occurred between the drug and polymer during the formation stages of nanoparticles by evaluating the alterations in frequency and intensity of the structures compared to FTIR signals of pure materials.[57]

Table 2. Particle size, polydispersity index, zeta potential, pH values and dirithromycin contents of Kollidon® SR nanoparticles prepared (n=3, mean ± standard error)

Code	PS (nm)	PdI	ZP (mV)	pH	DIR_S (%)	DIR_E (%)
Placebo KD	363.0±24.4	0.524±0.189	-19.6±4.1	5.04±0.01	-	-
KD1	329.6±6.8	0.425±0.080	-19.5±0.3	7.39±0.01	75.0±0.0	25.0±0.1
KD2	522.2±14.4	0.539±0.163	-25.5±0.1	7.90±0.00	55.6±0.1	44.4±0.1
Stability codes					**Total DIR loss (%)**	
Placebo KD-3/25	366.0±4.2	0.612±0.127	-20.6±1.1	5.54±0.10		
KD1-3/25	335.7±5.4	0.545±0.110	-21.5±0.2	7.42±0.01	0.18±0.02	
KD2-3/25	554.3±2.5	0.524±0.174	-26.1±1.1	7.98±0.02	1.2±0.01	
Placebo KD-3/40	373.0±5.1	0.628±0.199	-21.2±3.2	5.61±0.00		
KD1-3/40	348.6±7.8	0.614±0.092	-22.6±0.4	7.48±0.01	13.33±1.76	
KD2-3/40	562.4±6.3	0.654±0.183	-26.5±0.3	8.16±0.00	10.93±0.94	
Placebo KD-3/5	360.0±4.1	0.435±0.171	-20.1±4.1	5.74±0.02		
KD1-3/5	334.6±5.6	0.456±0.210	-21.4±0.3	7.56±0.01	1.69±0.90	
KD2-3/5	530.1±4.4	0.512±0.184	-26.4±0.1	7.88±0.01	1.77±0.22	

PS: Mean particle size, PdI: Polydispersity index, ZP: Zeta potential, DIR: Dirithromycin, DIR_S: Surface located DIR concentration, DIR_E: Encapsulated DIR concentration, Stability codes as x/y; x represents the storage month while y represents the storage conditions in °C

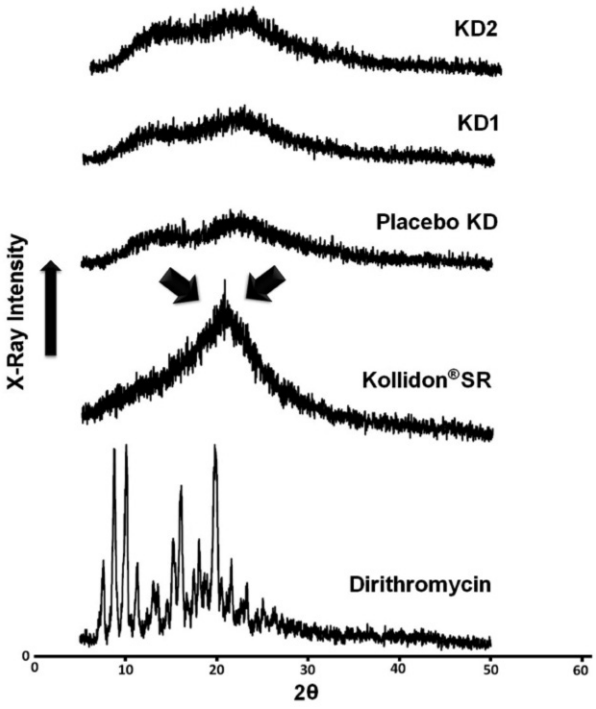

Figure 3. X-ray diffractometry spectra of dirithromycin, and nanoparticles prepared

KD: Kollidon®, Dirithromycin

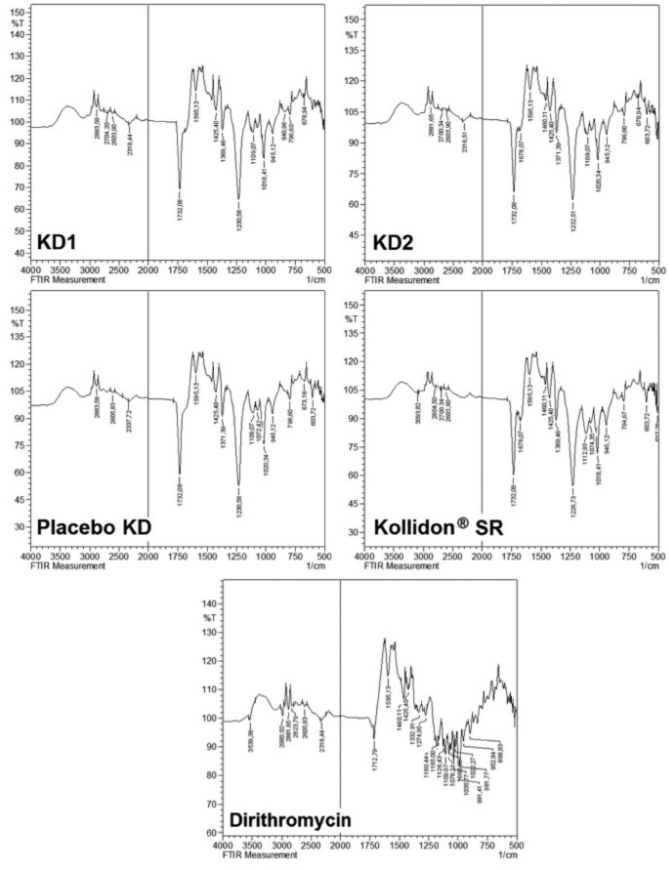

Figure 4. FTIR spectra of dirithromycin, and the nanoparticles prepared

KD: Kollidon®, Dirithromycin

Since the Kollidon® SR is a mixture of PVA and PVP, characteristic peaks were revealed for PVP N-C around 1230 cm^{-1} and PVA by C-O (stretch) at 1109 cm^{-1} and the C=O at 1732 cm^{-1} (Figure 4).[58,59]

Characteristic peaks of O-H streching at 3539 cm^{-1} observed in the pectrum of pure dirithromycin most probably due to the moisture content of the material.[60] C-H stretching (2980 cm^{-1}, 2881 cm^{-1}); C=O molecular vibration (1712 cm^{-1}) C=C (890-991 cm^{-1}) peaks were identified as the main groups (Figure 4).[20,61]

FTIR sptectra of pure materials were compared to KD1 and KD2 formulations and main signals were also detected in the spectra of formulations prepared therefore it showed that the formulation stages had no influence on the polymeric structure.

Intermolecular interactions of dirithromycin and Kollidon® SR was evaluated in this study also by ^{1}H-NMR.[62] Intensity of the peaks at 1-3 and 7-8 ppm ranges were increased slightly (marked with arrows) due to the presence of dirithromycin indicating the molecular distribution of dirithromycin within the polymeric structure (Figure 5).

Drug loading and encapsulation efficiency

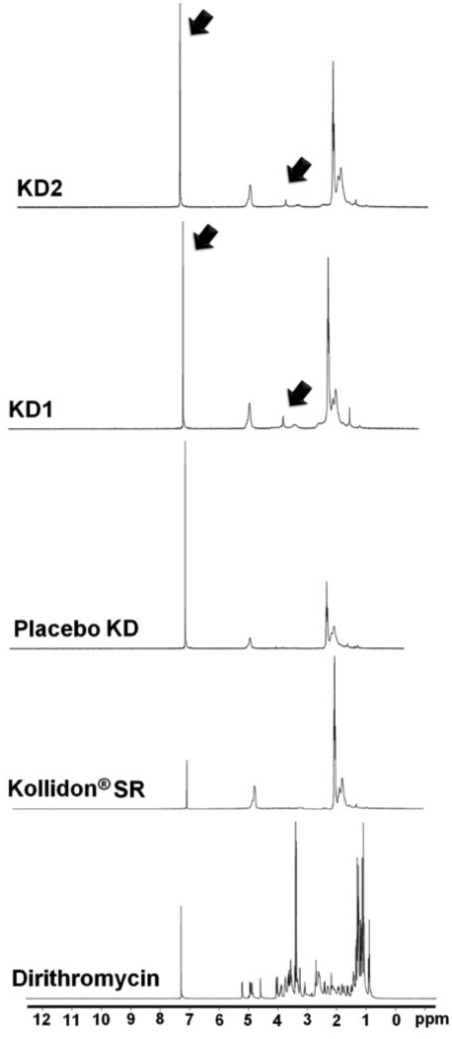

Figure 5. ^{1}H-NMR spectra of dirithromycin, and the nanoparticles prepared

As a result of HPLC method validation studies, linearity in methanol was y=1002.1x-1552.7 where r^2=0.997; accuracy was 97.8±1.9% for the concentrations of 5 µg/mL, precision was 4.9 and 5% for repeatability and reproducibility, respectively (n=3).

Dirithromycin amount of the nanoparticles prepared were presented at Table 2. Drug loading (%) and EE (%) were evaluated according to Equation 1 and Equation 2, respectively. Surface location was higher than the encapsulation for both formulations (75.0±0.0% and 55.6±0.1 for KD1 and KD2 respectively) (Table 2).

In vitro release study

Release profiles obtained for Kollidon® SR nanoparticles were given in Figure 6. The release profile of pure dirithromycin was used as a reference. According to the analysis results release rate of dirithromycin reached to 100% just after 2 hrs while the values for KD1 and KD2 formulations reached the highest points of 83.1% and 87.6% respectively after 6 hrs period (Figure 6). Initial burst releases were recorded from the nanoparticles as expected considering the surface location of the dirithromycin however drug releases were extended more than 3 fold which enhances the potential use of the nanoparticles for better treatment.

Realization of the *in vitro* release profiles with mathematical models that describes the dependence of release as a function of time gives valuable data about the *in vivo* release behaviour of the optimal delivery system.[63,64] Therefore *in vitro* release kinetics were also evaluated for the formulations prepared in comparison with pure Active pharmaceutical ingredient (API) using DDSolver Program (Table 3).[65] Best fitted models were selected considering the smaller Akaike information criterion (AIC) with higher adjusted R^2 values and the results were presented in Table 3.[63]

Pure dirithromycin's release kinetic was fitted to First Order which explains the release from the system where rate of drug release is concentration dependent.[66] Release kinetics of the formulations prepared were described by Baker-Lonsdale Model that describes the release kinetics from the matrixes of spherical in shape like many microparticle formulations (microcapsules and microspheres).[63,64] KD1 formulation also fitted to the Korsmeyer-Peppas Model which is a semi-empirical equation to describe drug release from polymeric systems when the release mechanism is not clear enough.[63] Considering the majority of the incorporated drug is located on the surface of KD1 formulation an initial burst effect was more obvious than KD2 formulation and therefore the difference between the release kinetics of the formulations can be attributed to the presence of more than one type of drug release of phenomenon was involved especially for KD1 formulation (Figure 6).[63] Kinetic models of both samples were also fitted to Weibull model which

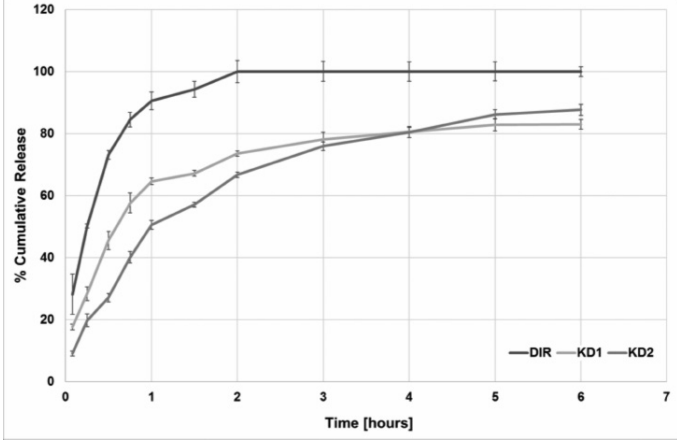

Figure 6. *In vitro* release profiles of dirithromycin from Kollidon® SR nanoparticles (n= 3, mean ± standard error)

DIR: Dirithromycin

Table 3. Best fitted *in vitro* release kinetic models of the dirithromycin and the formulations prepared

Code	Criteria	Zero order	First order	Higuchi	Korsmeyer-Peppas	Hopfenberg	Baker-Lonsdale	Weibull
DIR	k	0.025	0.003	0.56	0.045	0.00	0.000	α:0.00 β:0.001
	r^2	-0.003	0.001	0.000	-0.001	0.001	0.000	0.001
	AIC	0.113	0.061	0.100	0.106	0.092	0.096	0.050
KD1	k	0.020	0.001	0.043	0.056	0.00	0.000	α:0.001 β:0.000
	r^2	-0.001	0.001	0.001	0.001	0.00	0.001	0.001
	AIC	0.104	0.087	0.087	0.074	0.093	0.077	0.061
KD2	k	0.019	0.001	0.041	0.046	0.00	0.000	α:0.002 β:0.001
	r^2	0.000	0.001	0.001	0.001	0.001	0.001	0.001
	AIC	0.096	0.070	0.070	0.075	0.077	0.059	0.050

DIR: Dirithromycin, KD: Kollidon®

is an empirical model extensively used for fast and prolonged drug release profiles from matrix systems.[63,67] Weibull release kinetic model defined as the most applicable model almost all kinds of dissolution curves therefore has been subjected to some criticism.[64]

Evaluation of the cytotoxicity

Cytotoxicity of the nanoparticles prepared were evaluated by MTT method considering both dose and time dependancy on 3T3 mouse fibroblast cell lines.[68,69] At the highest dirithromycin concentration (0.25 µg/mL) the cell viability was found 83.36% and 63.36% after 24 and 48 hrs, respectively (Figure 7). Despite dirithromycin, no dramatic cell deaths were recorded for the Pure Kollidon® SR, Placebo KD, and KD1 formulations by valuing over 80% for all after the period of 48 hrs. However KD2 showed 88.01% and 63.48% cell viability after 24 and 48 hrs respectively considering the higher dirithromycin incorporation than KD1 (Figure 7). Analyses results showed that the concentration of the API has great influence on the cell viability. Achievement of the cell viability over 50% for all the formulations prepared showed that the formulations are safe to be applied to ocular the tissues.[33]

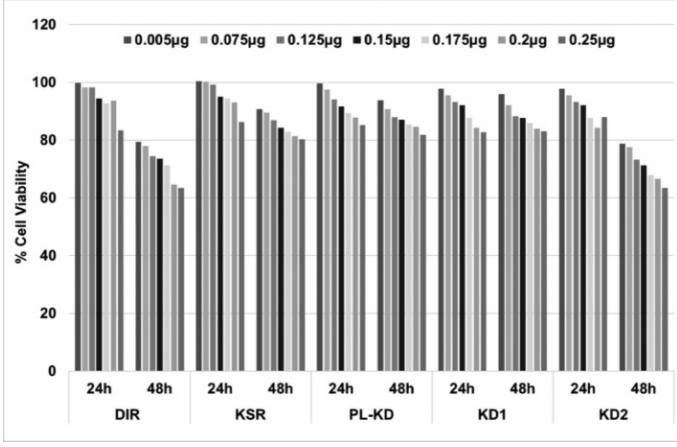

Figure 7. Cytotoxicity of dirithromycin, Kollidon® SR and nanoparticles prepared by MTT assay

DIR: Dirithromycin, KSR: Kollidon® SR, PL-KD: Placebo KD formulation, KD: Kollidon®, Dirithromycin

Microbiological assay

In vitro efficiency of the formulations were evaluated by Microbroth Dilution Method[35,36] on both gram positive and gram negative bacterias like *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) respectively. The MIC values of the formulations were presented in Table 4.

Analyses results demonstrated that KD1 has MIC values of 200 µg/mL and 100 µg/mL while KD2 showed MIC values of 100 µg/mL and 50 µg/mL on *S. aureus* and on *P. aeruginosa* respectively showing that formulations are more effective on gram negative bacterias. Since the constant amount of the samples were evaluated in Micro-Broth Dilution tests, comparable results were recorded for KD1 and KD2 considering the API concentration of KD2 which was nearly two fold than KD1 formulation. Even the API concentration of KD1 and KD2 were approx. 1/2.5 and 1/5 respect to pure API, same MIC values were achieved for KD2 formulation showing that polymeric carrier has also influenced the potency of the dirithromycin. Analyses results revealed that the incorporation of dirithromycin into the polymeric nanoparticles gives possibility to degrease the applied dose considering the efficacy of the formulation with low API concentration.

Table 4. Micro-broth dilution test results of the formulations prepared

Codes	*S. aureus ATCC 25923*	*P. aeruginosa ATCC 27853*
	MIC (µg/mL)	MIC (µg/mL)
KD1	200	100
KD2	100	50
Placebo KD	400	400
DIR	100	50

DIR: Dirithromycin, KD: Kollidon®, Dirithromycin

CONCLUSION

Sufficient treatment of the severe ocular infections mostly are being hampered because of the locational and structural features of the human eye. Therefore novel approaches are requisite for the enhancement of ocular bioavailability of the active materials especially for topical instillations. In this study dirithromycin incorporated Kollidon® SR-based nanoparticles were formulated for topical application. *In vitro* characteristic properties of the nanoparticles were evaluated in detail and stability of the formulations were evaluated during the storage period of 3 months.

Analyses results demonstrated that nanometer sized spherical particles were achieved by spray drying method. DSC and XRD analyses revealed the amorphous structure of the polymer which is prerequisite for the incorporation higher amounts of active agents due to the unorganized spaced within the polymeric network. FTIR studies showed no drug-polymer interaction while ^{1}H-NMR analyses revealed the presence of the active agent within the amorphous polymeric structure. Due to the high encapsulation efficacy of the particles, dirithromycin release could be extended up to 6 hrs which will enhance the therapeutic efficacy of the formulations prepared. Cytotoxic evaluation revealed the safety of the particles with relatively high cell viability data. Microbiological experiments revealed the potency of the formulations prepared on both gram positive and gram negative bacterias.

Therefore analyses results can be concluded as Kollidon® SR-based nanoparticles are effective carrier candidates for the topical ocular application of dirithromycin. However *in vivo* analyses results are required for the final decision to be made.

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