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Cefotaxime Combined Ellagic Acid in a Liposomal Form for More Stable and Antimicrobial Effective Formula

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Abstract The aim of this study is loading of cefotaxime (CXM) and ellagic acid (EA) in a liposomal formula to enhance CXM corneal permeability, stability and antimicrobial activity, thin film hydration method used to form CXM- EA liposomes, particle size, zeta potential, scan electron microscope image, CXM release, drug stability and antimicrobial activity were tested. CTX entrapped in CXM – EA liposomes was 42.1 ± 3.2%, ellagic acid content was 72.1 ± 3.1%, particle size was 251.7 ± 1.2 nm, and Zeta potential was 12.4 ± 3.1 mV with a polydispersity index of 0.34 ± 0.21.In concern to CXM released, it was a dramatic rapid diffusion of raw CXM (~88%) after 1 hour, however, CXM released from CXM –EA liposomes (~50 %), after 2 h, raw CXM was completely dissolved in the buffer medium, but it takes about 8 h to be completely released to the buffered medium. Stability study was carried out among 14 days at room and refrigerator temperatures, raw CXM was expired after 7 days while the formulated CXM content was (~93 %) and (~96 %) for room and refrigerator temperature respectively, finally, antimicrobial activity was carried out against two gram positive and two gram negative microorganisms, data revealed that ellagic acid potentiates the antimicrobial activity of CXM.

Keywords: *cephalosporins, eye infection, cornea, nanoparticles, vesicles, antimicrobial effect*

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1. Introduction

CXM is one of a β-lactam antibiotic in the third-generation class of cephalosporins [1], CXM is active against numerous gram positive and gram negative bacteria as Haemophilus influenza, Proteus mirabilis, Neisseria gonorrhoeae, and Streptococcus pneumonia so used mainly for treatment respiratory tract infections, intra-abdominal cavity, skin, central nervous system and many other types of infection [2]. Ophthalmic drug delivery facing some challenges to achieve effective treatment, like static and dynamic barriers and efflux pumps that impede drug delivery, particularly to the posterior eye segment however, the ocular treatment allows lower incidence of systemic drug side effects and drug–drug interactions that are common during systemic treatment [3]. Liposomes are artificial vesicles comprising of an external bilayer of lipids encasing a focal watery center [4,5]. Due to this novel structure, they can capture both the hydrophilic and hydrophobic medications [6,7]. Other than this specifically preferred standpoint, liposomes are likewise biocompatible, biodegradable and non-poisonous [8]. They are of adequate adaptability to permit combination in different sizes and can be planned as eye drops, gels and treatments for topical conveyance. Be that as it may, the ordinary liposomes had the burden of being insecure, getting collected and were powerless to phagocytosis [9,10]. With a specific end goal to overcome these issues and to accomplish delayed and focused on medication conveyance, a radical new era of liposomes has developed [11]. As the territory of new era liposomes stays unexplored for topical medication conveyance. Another challenge facing CXM to be used in form of eye drop is its stability in the aqueous medium, which could be resolved by adding a potent antioxidant to the formula that protects CXM from the oxidation [12]. EA is a phenolic acid present in many plant extracts. The phenolic acids and flavonoids are shaping a polyphenolic intensify that broadly spread in plants, and they named by nutraceuticals. Studies alluded to that polyphenols have organic exercises toward various diseases EA was accounted for to shield creature from Salmonella typhymurium [13]. The aim of this study was to combine CXM with EA in form of liposome to enhance and sustain the ocular drug delivery.

2. Materials and Methods

Cefotaxime sodium and ellagic acid were purchased from Xian Warton Biological technology company (Shaanxi, China), α-phosphatidylcholine and cholesterol were purchased from Sigma-Aldrich (Detsenhofen, Germany), Tween 20 was purchased from Fluka (Düsseldorf, Germany), All other reagents and chemicals were of analytical grade.

2.1. CXM-EA Liposomes Preparation

CXM-EA liposomes were prepared by thin film hydration method [14]. 200 mg of Cholesterol is chemically combined with 150 mg of α-phosphatidylcholine to form the outer layer. 20 mg EA was added to 20 ml of chloroform then added to α-phosphatidylcholine and cholesterol layer, chloroform was evaporated by a rotary evaporator to form a thin film. The lipid film is hydrated with an aqueous buffer (PH, 7) containing 50 mg of a surfactant (tween 20) and 100 mg of CXM sonicated for 10 minutes resulting in liposome formation.

2.2. Characterization of CXM-EA Liposomes

2.2.1. Evaluation of CXM–EA Liposomes Morphology by Scanning Electron Microscopy (SEM)

Surface morphology of the CXM-EA liposomes was studied using scanning electron microscope (Philips XL30, Eindhoven, Netherlands). To avoid elongation as a result of lyophilization process or colligation of the prepared liposomes , 15 % w/v mannitol solution was added to the precipitated CXM-EA liposomes with ratio (1: 1),the role of mannitol solution is a cryoprotectant, the solution was freezed at -80°C, finally subjected to freeze-drying for 48h using ((Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). The lyophilized sample was coated with gold before investigation.

2.3. CXM-EA Liposomes Size and Zeta Potential Measurement

The size and zeta potential of CXM-EA liposomes were measured by photon correlation spectroscopy using a Zetasizer (Zetatrac, Mongomeryville, PA, USA). Samples were appropriately diluted with the distilled water (10 – folds) dilution before measurement to get an optimum of 50–200 kilo counts per second (kcps), Sample was measured three times.

2.4. Entrapment Efficiency Measurement

Indirect method was used to measure entrapped CXM and EA, the prepared liposomes was subjected to ultracentrifugation at 50,000 rpm for 50 minutes using Hitachi ultracentrifuge CP100WX, Hitachi, Koki Co., Ltd. (Hitachi, Tokyo, Japan) [15]. The supernatant which contains free CXM and EA was filtered by cellulose acetate membrane (0.2 µm) then diluted with distilled water, CXM and EA were measured by the high-performance liquid chromatography (HPLC) system.

The entrapment efficiency was calculated using the following equation:

$$\text{Entrapment efficiency (\%)} = \frac{D_{total} - D_{free}}{D_{total}} \times 100$$

Where D_{total} is the total concentration of the drug loaded and $D_{free\ drug}$ represents the concentration of the free drug in the supernatant., Paleti HPLC method [16] was modified to determine both CXM and EA using HPLC system Agilent 1,200 infinity series, equipped with an ultra violet (UV) diode array detector and an automatic sampling system, Agilent Technologies (Santa Clara, CA, USA).

2.5. CXM - EA liposomal Release Study

To compare the diffusion of CXM in the form of liposomes with pure CXM, volume of liposomal solution contains 20 mg of CXM and the same pure CXM weight was added to the same volume of distilled water , each sample was placed in donor chamber of automated Franz diffusion cell apparatus (Microette Plus™; Hanson Research, Chatsworth, CA, USA) [17], after that covered with a synthetic nylon membrane of pore size 0.45 µm. phosphate buffer with pH 7.4 was used as a medium for this experiment and placed in the receptor chamber, the temperature was kept at 32°C ± 0.5°C and the stirring rate was adjusted at 400 rpm. Samples were withdrawn automatically from the receptor chamber with time schedule 0.5, 1, 2, 4, 6 and 8 h and replaced the buffer medium, samples were analyzed by the same HPLC method which mentioned previously in EE% paragraph.

2.6. Stability Study

Stability study was conducted for 14 days on pure CXM and CXM-EA liposomes with concentration (0.5 mg/ml) for both solutions at two temperatures [18]. The tested solutions were stored at different temperatures like room temperature (22°C), and refrigerator (4°C). Samples were withdrawn at different time intervals 0, 1, 2,7,10, 14 days and CXM was quantitated by the same HPLC method for EE %.all samples were triplicates and represent with mean ± SD.

2.7. Antimicrobial Evaluation

The antimicrobial activity of CXM-EA liposomes and pure CXM were tested against standard strains of four bacteria. They were obtained from the microbiology laboratory, King Abdulaziz University Hospital, Jeddah, KSA. These strains included Gram positive bacteria: Staphylococcus. Aureus ATCC 29213 and Bacillus subtilis ATCC 6633 and Gram negative bacteria: Escherichia coli ATCC 35218 and Pseudomonas aeruginosa ATCC 27853.

Agar diffusion method was used to evaluate the antimicrobial activity of CXM liposomes in comparing with raw CXM ,the method, as described previously [19], Petri dishes (150 mm) containing 25-mL Muller-Hinton agar containing 1 mL bacterial culture of number (1 × 106 CFU/mL). Each strain was immunized independently. A 10 mm measurement gaps were made in the seeded agar plates. The openings were then loaded with 200-µL of the raw CXM and CXM-EA liposomes solutions with concentration 0.5 mg /ml CMX for each sample. Dishes were then incubated for 24 h at 37°C, Inhibitory zone was characterized as the nonattendance of bacterial development in the territory encompassing the gaps. The inhibitory zone was measured utilizing a caliper. All specimens were triplicates and expressed with mean ± SD. Analysis of variance (ANOVA) calculated by Tukey's pairwise correlations test; the confidence level was set at P,0.05 GraphPad Prism 6 (GraphPad Software, San Diego, CA).

3. Results and Discussion

EA was previously combined with other drugs in form of liposomes [20,21], in our study some trials were carried out to reach the most stable, smaller size with highest EE %, CTX (%EE) of the prepared CXM – EA liposomes was 42.1 ± 3.2%, EA content was 72.1 ± 3.1%, particle size was 251.7 ± 1.2 nm, and Zeta potential was 12.4 ± 3.1 mV with a polydispersity index of 0.34 ± 0.21.Data revealed that the relatively low EE% of CXM , That could be rationalized by the free solubility of CXM in water, although this low EE%, but it is considered high EE% if compared with other published report with similar water solubility [22], that could attribute to α-phosphatidylcholine which decreases interfacial tension between the aqueous and organic phase leading to a homogeneous and stable emulsion. This then enhances CTX entrapment in the CTX-ALA liposomes [23]. Cholesterol could exclude EA because of competition between cholesterol and EA for packing space within the vesicular bilayer. Tween 20 found to have a significant effect on CXM EE%. This could be attributed to the solubility of both CXM and EA in tween 20 and in water. The ultrasonication may enhance the mobility of tween 20 so, lipid molecules rearranged that allows for higher EE% of the CXM [24]. The scanning electron microscopy of the CXM-EA liposomes image (Figure 1), shows spherical vesicles with a circular shape and homogenous unilamellar edges. The effect of organic solvent on liposomal shape is as reported previously by Ahmed, gradual evaporation with rotation lead to phospholipid in the form of a tightly packed bilayer with a closed configuration [25].

As shown in Figure 2 Comparative permeation profiles of raw CXM which compared with CXM released from CXM-liposome. There was a dramatic rapid diffusion of CXM (~88%) after 1 hour, however, CXM released from CXM –EA liposomes (~50 %), after 2 h, raw CXM was completely dissolved in the buffer medium, but it takes about 8 h to be completely released to the buffer medium. Initially stage, burst effect, it is usually due to the fast release of CXM entrapped on the CXM-EA liposomes surface. After this stage, slow release of CMX from the liposomes. The slow release after the initial stage is predicted as a result of deeper entrapped CXM in the core matrix of the liposomes body. CXM in the core of the liposomes has a longer diffusion route to reach the surface compared to CXM entrapped on the surface. The release of CXM form core matrix of the liposomes is affected by factors that control CXM release kinetics from the fatty matrix. These factors are; liposomes size, CXM dissolution/diffusion rate, CXM water solubility, and the matrix erosion/ degradation rate. In addition, the hydrophobic nature of the α-phosphatidylcholine and cholesterol augments the delay of water penetration [26,27,28].

3.1. Stability Study

Table 1 shows stability data for both CXM and CXM – EA liposomes, the observed results showed great stability for CXM – liposomes in comparing with raw CXM, after 14 days raw CXM was degrade to (~80 %) at 22°C however, the formulated CXM was (~93 %), we can conclude that raw CXM was expired after 7 days which agree with data [29], while liposomal CXM exceeded 14 days for both temperatures.

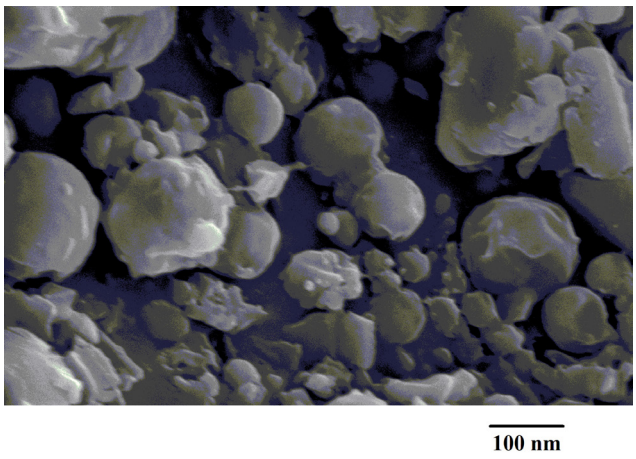

Figure 1. SEM image of CXM-EA liposome

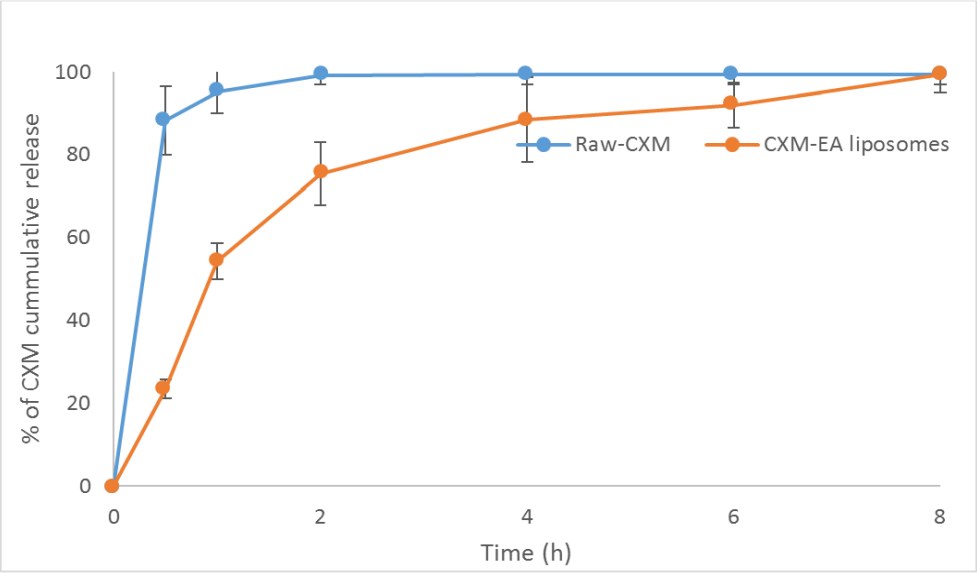

Figure 2. CXM cumulative release % of raw drug and CXM-EA liposomes

Table 1. Chemical Stability of raw CXM and CXM-EA liposomes at 4 and 22°C temperatures

	Temp.	0 day	1 day	2 day	7 day	10 day	14 day
Raw CXM	4ºC	99.3 ± 1.2	97.1 ± 1.1	96.3 ± 3.1	91.3 ± 3.5	90.3 ± 2.5	87.3 ± 3.3
	22ºC	99.3 ± 2.1	99.3 ± 1.2	95.3 ± 2.2	88.2 ± 2.2	83.3 ± 2.2	80.1 ± 2.1
CXM – EA liposomes	4ºC	99.8 ± 1.1	99.1 ± 1.1	98.3 ± 1.6	97.3 ± 1.8	97.2 ± 2.1	96.4 ± 2.2
	22ºC	99.1 ± 1.2	99.1 ± 1.4	97.3 ± 2.1	96.3 ± 1.2	95.3 ± 2.8	93.3 ± 2.3

* Significant effect between the two groups.

Table 2. Antibacterial activities of formulae studied

	Zone diameter (mm)			
	Gram-positive bacteria		Gram-negative bacteria	
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*
Raw _CXM	50 ± 2.1	43± 2.3	51± 3.1	48± 3.2
CXM-EA liposomes	57± 1.3*	45± 1.2	54± 2.1	58± 1.4*

* Significant effect between the two treatments against gram positive and gram – negative bacteria.

3.2. Antimicrobial Activity

Antimicrobial activity was carried out to evaluate the enhancement of CXM activity after incorporation with EA, data in Table 2 revealed that CXM-EA liposomes have wider inhibition zone against all bacteria types, but in case of Staphylococcus aureus and Pseudomonas aeruginosa the enhancement was significant in comparing with raw CXM, previous reports confirmed the antibacterial activity of EA, it has moderate to strong toxicity against many types of bacteria and pathogens, this toxicity is due to hydrolysis of ellagitannin then lactonization of hexahydroxydiphenic acid [30]. Other bactericidal substances found in EA like β-glycyrrhetinic acid, sorgoleone, and wogonin which have antimicrobial activity against Gram-positive and Gram-negative bacteria [31], moreover, EA microbicidal compounds do not affect on anti-microbial activity of some antibiotics [32], another reason for improving CXM-EA liposomal antimicrobial toxicity is the liposomal form which contains surfactant and fatty nature compounds which increase the solution viscosity and stability [32]. ellagitannins as antimicrobial agents probably, coupled with protein in bacteria walls, such as that of Bacillus, Staphylococcus, and Salmonella. Other bactericidal activity could be by inhibition of methicillin-resistant Staphylococcus aureus strains, last it could be due to the ability to inhibit gyrase activity which is associated with the cleavage of the DNA strand during the replication process [33].

4. Conclusion

CXM and EA were successfully loaded on a liposomal form using thin layer hydration method. The produced liposomes were in nano size, spherical in shape with gradual CXM release which enhances the therapeutic efficacy, moreover enhanced CXM stability and antimicrobial activity, this will enhance patient tolerability and convenience. My data suggest that combined CXM and EA in a liposomal form could enhance CXM therapeutic efficacy but still in need of more clinical studies.

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