ORİJİNAL MAKALE / ORIGINAL ARTICLE

Balıkesir Sağlık Bilimleri Dergisi / BAUN Sağ Bil Derg
Balıkesir Health Sciences Journal / BAUN Health Sci J
ISSN: 2146-9601- e ISSN: 2147-2238
Doi: https://doi.org/10.53424/balikesirsbd.1262051

The Preparation of Liposomal Formulations of Gentamicin and Ceftiofur Used in Veterinary Medicine

Hasan SUSAR[1], Murat ÇELEBİ[2], Çağla ÇELEBİ[3], Özlem ÇOBAN[4], Hüseyin ŞEN[3], İzzet KARAHAN[1]

[1] Balıkesir University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology
[2] Balıkesir University, Savaştepe Vocational School, Department of Laboratory and Veterinary Health
[3] Balıkesir University, Health Sciences Institute, Department of Pharmacology and Toxicology
[4] Karadeniz Technical University Faculty of Pharmacy, Drug and Pharmaceutical Technology Application and Research Center, Department of Pharmaceutical Technology

Geliş Tarihi / Received: *08.03.2023,* ***Kabul Tarihi / Accepted:*** *22.05.2023*

ABSTRACT

Objective: The aim of this study is to produce liposomal drugs that are scarcely found in veterinary medicine. Therefore, the current study was designed to produce liposomal formulations of ceftiofur and gentamicin for veterinary use and to perform their quality control studies. **Materials and Methods:** The Bangham Method was chosen for the preparation of liposomal formulations of gentamicin and ceftiofur. **Results:** The particle size, polydispersity index and zeta potential were found to be 3934.67 nm, 0.471, -10.3 mV for Ceftiofur-1 coded formulation, 4573.0 nm, 0.308, -9.9 mV for Ceftiofur-2 coded formulation, 479.4 nm, 0.437, -44.8 mV for Ceftiofur-3 coded formulation, respectively. The particle size, polydispersity index and zeta potential were found to be 5185.67 nm, 0.599, -6.5 mV for Gentamicin-1 coded formulation, 4228.0 nm, 0.505, -7.8 mV for Gentamicin-2 coded formulation, 2138.67 nm, 0.565, -6.5 mV for Gentamicin-3 coded formulation, respectively. The encapsulation efficiency of liposomal formulations containing ceftiofur; 82.85%, 95.74%, and 92.06% was found for ceftiofur-1, ceftiofur-2 and ceftiofur 3, respectively. **Conclusion:** The liposomal formulations of ceftiofur and gentamicin were successfully prepared and their quality control studies were carried out. It was concluded that pharmacokinetic/pharmacodynamic studies should be performed to evaluate the efficacy of liposomal formulations.

Keywords: Drug, Formulation, Liposome, Veterinarian.

Veteriner Hekimlikte Kullanılan Gentamisin ve Seftiofur'un Lipozomal Formülasyonlarının Hazırlanması

ÖZ

Amaç: Bu çalışmanın amacı, veteriner hekimlikte çok az bulunan lipozomal ilaçların üretilmesidir. Bu nedenle mevcut çalışma, veteriner kullanım için seftiofur ve gentamisinin lipozomal formülasyonlarını üretmek ve kalite kontrol çalışmalarını yapmak hedefleriyle tasarlanmıştır. **Gereç ve Yöntem:** Gentamisin ve seftiofur'un lipozomal formülasyonlarının hazırlanması amacıyla Bangham Metodu tercih edildi. **Bulgular:** Sonuç olarak; seftiofur-1 lipozomunun partikül boyutu 3934.67 nm, polidispersite indeksi 0.471, zeta potansiyeli -10.3 mV'dir; Ceftiofur-2 lipozomunda; parçacık boyutu 4573.00 nm, polidispersite indeksi 0.308, zeta potansiyeli -09.9 mV; Ceftiofur-3 lipozomlarında; parçacık boyutu 479.40 nm, polidispersite indeksi 0.437, zeta potansiyeli -44.8 mV ölçülmüştür. Gentamisin-1 lipozomunda; parçacık boyutu 5185.67 nm, polidispersite indeksi 0.599, zeta potansiyeli -06.5 mV; Gentamisin-2 lipozomunda; parçacık boyutu 4228.00 nm, polidispersite indeksi 0.599, zeta potansiyeli -07.8 mV; Gentamisin-3 lipozomunda; parçacık boyutu 2138.67 nm, polidispersite indeksi 0.565, zeta potansiyeli -06.5 mV ölçülmüştür. **Sonuç:** Seftiofur ve gentamisinin başarılı bir şekilde lipozomal formülasyonları hazırlandı ve kalite kontrol çalışmaları gerçekleştirildi. Lipozomal formülasyonların etkinliklerinin değerlendirilmesi için ise farmakokinetik/farmakodinamik çalışmaların yapılması gerektiği kanaatine varıldı.

Anahtar Kelimeler: İlaç, Formülasyon, Lipozom, Veteriner.

Sorumlu Yazar/Corresponding Author: Hasan SUSAR, Balıkesir University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Balıkesir, Türkiye.
E-mail: hasan.susar@balikesir.edu.tr

Bu makaleye atıf yapmak için / Cite this article: Susar, H., Celebi, M., Celebi, C., Coban, O., Sen, H., Karahan, I. (2023). Preparation of liposomal formulations of gentamicin and ceftiofur used in veterinary medicine. *BAUN Health Sci J, 12*(3),554-559. *https://doi.org/10.53424/balikesirsbd.1262051*

INTRODUCTION

Drug resistance is a major problem in human and veterinary medicine worldwide. It reduces the effectiveness of many conventional antimicrobial medicines for treating infections (Gupta et al., 2019). In fact, eradicating resistant pathogens is a important challenge and requires a higher range of antibacterial agent at the site of action. Administering such high doses of antibiotics can be highly toxic or even fatal (Gonzalez et al., 2019). To overcome the ongoing problem, numerous researchers focused on two solutions; search for new antibacterial agents and development of drug delivery methods that improve the effectiveness of current antimicrobial agents (Shen et al., 2017). Encapsulation of antibiotics in liposomes improves their targeted effects and decreases their off-target effects. To maintain the efficacy of antibacterial agents, it protects cells from elimination of the body's immun system and the toxic effects of the loaded drug. Therefore, liposomes are considered efficient as a drug delivery system to improve the biocompatibility of antimicrobial agents (Al-Darraji et al., 2020; Saddiqi et al., 2022).

Nanotechnology is a promising topic of science that comprises the advancement and generation of a wide range of nanomaterials. In the past few decades, the field of nanotechnology has grown exponentially. A wide range of nanoparticles such as liposomes, dendrimers, micelles, and organic nanoparticles are applied used as a drug delivery system. Liposomes are the most widely used and well-known nanoparticles among various types of nanomaterials in nanomedicine because they are biodegradable due to their high biocompatibility and biodegrability and show less toxic effects for *in vivo* use. These unique characteristic properties of liposomes result from their high similarity to the cell membrane in terms of both structure and components, facilitation of effective interaction between liposomes and the cell membrane and as a result increasing yield and cellular uptake. Liposomes are defined as spherical vesicles composed of one or more phospholipid bilayers surrounding an aquatic core. It can improve the pharmacokinetics/pharmacodynamics of antimicrobial drugs and reduce their toxic effects (Beltrán-Gracia et al., 2019; Kim & Jeong, 2021).

Ceftiofur is a third-generation cephalosporin antibiotic approved for animal health use only for the treatment of systemic infections caused by susceptible pathogens in horses, cattle, dogs, sheep and goats. It has a wide range of uses against diseases caused by Gram (+) and Gram (-) bacteria in milking cows, notably because it does not leave drug residues in milk (Cavanagh, 2012). It has highly effective *in vitro* against pathogenic bacteria such as *Pasteurella spp., Haemophilus spp., Salmonella spp., Mannhemia spp., and Actinobacillus* spp. (Hornish & Kotarski, 2002).

Gentamicin is an aminoglycoside antibiotic that has bactericid activity against many Gram (+) positive and Gram (-) negative organisms. In the group of aminoglycosides, following amikacin, it has the broadest spectrum and the highest antibacterial effect. Depending on the concentration, they usually have a rapid bactericid effect against a wide variety of aerobic bacterial strains. Due to aminoglycosides are polar substances, they enter bacterial cells only by active transport, therefore, not active against aerobic or anaerobic bacteria under anaerobic conditions. Because of these properties, they are very few absorbed from gastrointestinal tract. They across placenta, can accumulate in fetal plasma and amniotic fluid, but cannot across the blood-brain barrier. They have a limited distribution in the body's extracellular fluid and their transition into mammalian cells is difficult. It is only slightly bound to plasma proteins. It is excreted unchanged from the body through the kidneys. Depending on the application, there is a 5-25% risk of developing nephrotoxicity. Therefore, they are narrow spectrumed drugs. Nephrotoxicity is directly proportional to the dose and duration of drug use and is generally reversible (Ali et al., 2011; Chou et al., 2015; Kayaalp, 2018).

When all this knowledge is evaluated, it is also important to prepare liposomal forms of drugs such as ceftiofur and gentamicin. In this study, it is aimed to investigate how to prepare liposomal forms of these drugs under laboratory conditions.

MATERIALS AND METHODS

Procedures

Used materials; phosphotiditylcholine (L-α-phosphatidylcholine, Sigma-Aldrich), cholesterol (Acros Organics, Belgium), gentamicin sulfate (Acros Organic 455310010), ceftiofur (Acros Organic 465890010), chloroform, methanol, dimethylsulfoxide (Sigma-Aldrich), phosphate buffer tablet (Oxoid-BR0014G, UK), Rotary evaporator (Isolab), Malvern Zetasizer NanoZS, UV-VIS Spectrophotometer (UV1900i) and glass laboratory equipments.

Liposomes were prepared by thin layer hydration technique (Bangham, et al., 1965). The active ingredients ceftiofur, gentamicin, phospholipid (PC, egg phosphotidylcholine), cholesterol (CL) to be liposomized were weighed on a sensitive balance and placed in beakers. Chloroform: methanol was added in a 1:1 ratio. The substances in the beaker were dissolved with gentle wrist movements until complete clarification was achieved. This mixture was poured into a 100 mL volatile flask and attached to the rotary evaporator apparatus. To obtain the lipid film; in a rotary evaporator at 37° C., the mixture was evaporated for 15 minutes at a speed of 250 rpm. As a result of these processes, a lipid film layer was obtained. To hydrate the resulting dry lipid film, 10 mL of distilled water was added to the evaporating flask and rotated without vacuum. The mouth of the glass flask was sealed with parafilm and vortexed. It was placed in an ultrasonic bath for 20 minutes to reduce the particle size. The final mixture was placed in tubes for centrifugation and stored in a refrigerator at 4°C with sealed tightly.

Centrifugation was performed at 16,500 rpm for 40 minutes. The collapsing liposomal portion and the aqueous portion were placed in separate test tubes and refrigerated at 4°C for further analysis. The amounts and lipid/cholesterol ratios of all ingredients in the formulations, the amounts of drug molecules, solvents and dispersion liquids are shown in Table 1.

The particle size, polydispersity index and zeta potential of liposomes were measured using Malvern Zetasizer Nano-ZS (United Kingdom, UK) equipped with dynamic light scattering (DLS) and electrophoretic light scattering techniques (Çoban et al., 2021). For this purpose, the liposomes precipitated by centrifugation were mixed with 1 ml of distilled water and dispersed. 120 µL of these samples were taken and placed in the measuring cuvette and 1880 µL of distilled water were added. All measurements were performed at 25± 0.1 ºC.

The encapsulation efficiency (EE) of ceftiofur liposomes was calculated using the following formula (Eq.1). The encapsulation efficiency of ceftiofur was measured at 292 nm (Çoban et al., 2020).

$$EE\ (\%) = \frac{\text{Total drug} - \text{Free drug in supernatant}}{\text{Total drug}} x100 \quad \textbf{Eq.1}$$

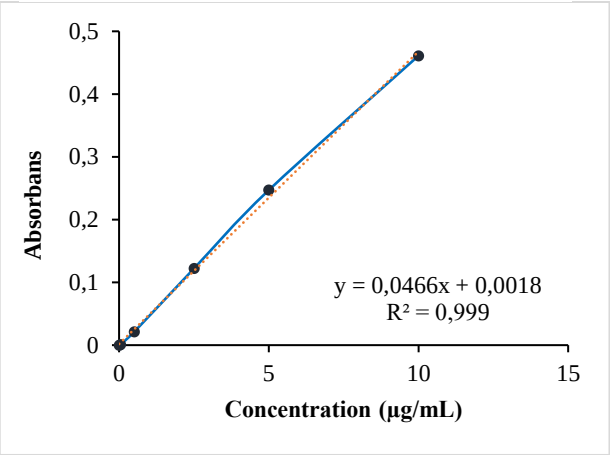

Figure 1. Calibration curve of ceftiofur.

Table 1. Amount of substance in liposome preparation.

No	Formulation name	Lipid cholesterol		Active drug	Amount Of solvent		Amount Of dispersion
		PC : CL (mg)		(mg)	Chloroform (ml): Methanol (ml)		Distilled Water (ml)
1	Ceftiofur-1	3 (50.5)	1 (19.8)	25.5	1 (5.0)	1 (5.0)	10
2	Ceftiofur-2	2 (60.0)	1 (29.8)	25.2	1 (5.0)	1 (5.0)	10 (PBS)
3	Ceftiofur-3	3 (32.0)	1 (12.0)	31.0	1 (5.0)	1 (5.0)	10
4	Gentamicin-1	2 (50.2)	1 (30.2)	49.9	1 (5.0)	1 (5.0)	10
5	Gentamicin-2	1 (50.0)	1 (49.5)	25.1	1 (7.5)	1 (7.5)	10
6	Gentamicin-3	1 (50.2)	1 (50.0)	25.3	1 (7.5)	1 (7.5)	10

SEM (Scanning Electron Microscope) analysis

The scanning electron microscope used to take pictures is a JEOL Neoscope brand JCM-5000 SEM.

Before the images were taken, approximately 0.02 g of gentamicin and ceftiofur in the liposomal formulation were weighed and placed on a bidirectional carbon tape. First, gold plating was performed by applying a vacuum of 8 x 10 -1 mbar/Pa and a voltage of 10 mA in the quorum coater. Gentamicin was examined in SEM at 2000x and 1500x magnification (Figure 2). Ceftiofur was examined in the SEM at 1000x magnification (Figure 3).

Statistical analysis

The statistical analysis of the experimental results was performed by Student's t-test using the GraphPad Prism 5.0 and all data were expressed as the mean standard deviation (SD). P values less than 0.05 were considered a statistically significant value.

RESULTS

This research was conducted to determine the physical properties of the active ingredient that can affect drug performance and development. Our study was conducted alone or to determine the relationship with excipients to ensure that the drug is of good quality in terms of physical and chemical properties.

Looking at Table 2, the particle size, zeta potential and polydispersity index results can be seen. The particle size, polydispersity index and zeta potential were found to be 3934.67 nm, 0.471, -10.3 mV for Ceftiofur-1 coded formulation, 4573.0 nm, 0.308, -9.9 mV for Ceftiofur-2 coded formulation, 479.4 nm, 0.437, -44.8 mV for Ceftiofur-3 coded formulation, respectively.

The particle size, polydispersity index and zeta potential were found to be 5185.67 nm, 0.599, -6.5 mV for Gentamicin-1 coded formulation, 4228.0 nm, 0.505, -7.8 mV for Gentamicin-2 coded formulation, 2138.67 nm, 0.565, -6.5 mV for Gentamicin-3 coded formulation, respectively.

The encapsulation efficiency of liposomal formulations containing ceftiofur; 82.85%, 95.74%, and 92.06% was found for ceftiofur-1, ceftiofur-2 and ceftiofur 3, respectively. In liposomal formulations containing gentamicin, measurements of the encapsulation efficiency could not be performed because the results of the drug's wavelength scans could not be found in the UV-VIS spectrophotometer.

Table 2. Measurements of liposomal formulations.

No	Formulation Name	Particle Size Mean±SD (nm)	Polydispersity Index Av. Mean±SD	Zeta Potential Mean±SD (mV)
1	Ceftiofur-1	3934.67±463.32	0.471±0.009	-10.3±0.3
2	Ceftiofur-2	4573.00±524.00	0.308±0.197	-9.9±0.8
3	Ceftiofur-3	479.40±12.13	0.437±0.022	-44.8±0.6
4	Gentamicin-1	5185.67±472.48	0.599±0.044	-6.5±0.2
5	Gentamicin-2	4228.00±315.98	0.505±0.099	-7.8±0.0
6	Gentamicin-3	2138.67±34.32	0.565±0.050	-6.5±0.3

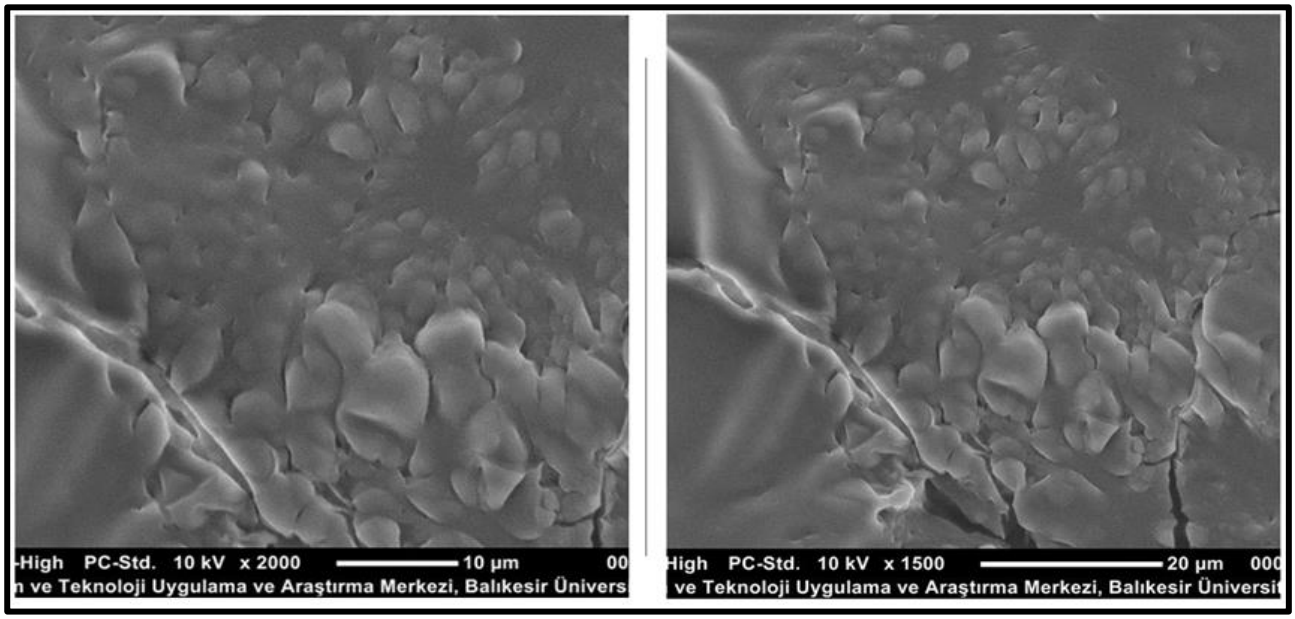

Figure 2. Gentamicin imaging under SEM.

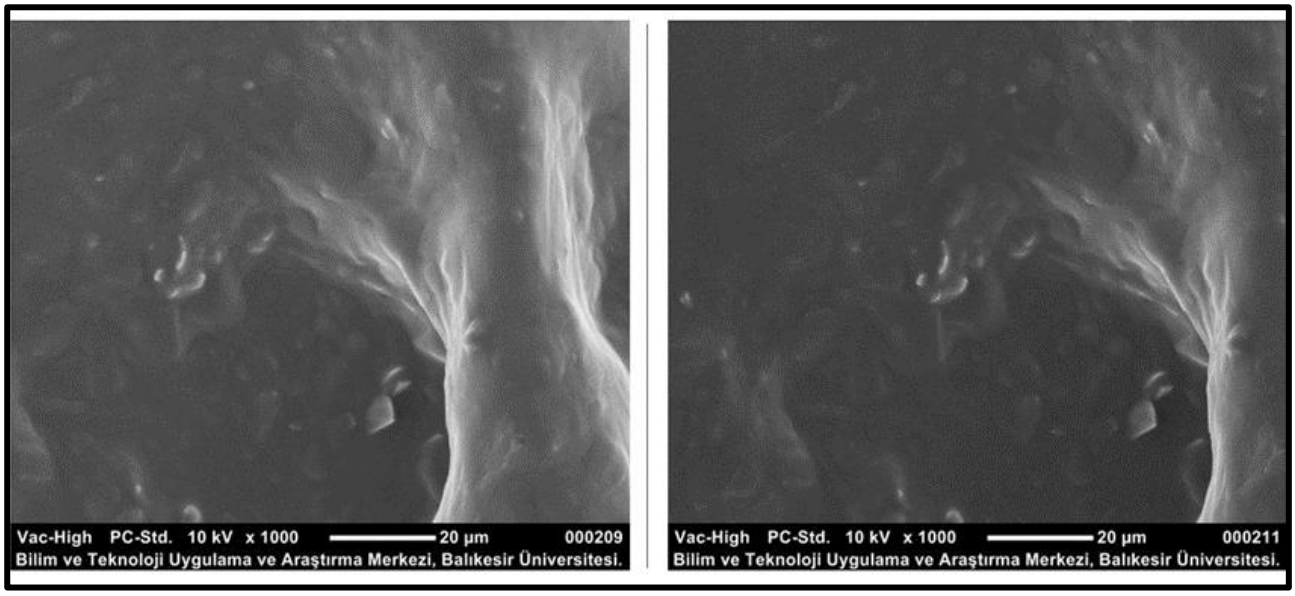

Figure 3. Ceftiofur imaging under SEM.

DISCUSSION

Particle size measurements in liposomes; it is an important quality control parameter to provide information about the physical properties and stability of the particles and to predict the stability of the particles in the body and their behavior in plasma and blood. Polydispersity index (PDI) measurements performed in liposomal formulations indicate the distribution of molecular weight in a given sample. A PDI value close to 1 indicates high particle size heterogeneity and no uniform size distribution. Although the particle size of liposomes varies depending on the oil composition and manufacturing processes and the properties of the encapsulated substance, the presence of cholesterol in the liposomal system increases the particle size (Grazia Calvagno, 2007; Pattni et al., 2015; Wagner et al., 2006). In our study, it was hypothesized that cholesterol-induced particle size might increase.

It is stated that the intended uniform particle distribution in liposome preparation depends on the purpose of usage. The researchers found that the particle size distribution was homogeneous in liposome preparation made by the extrusion process (Beltrán-Gracia et al., 2019; Pattni et al., 2015). It was found that the liposome samples obtained did not have a homogeneous particle distribution. In another study that generated liposomal ceftiofur, an encapsulation efficiency was found 57.2%. They reported that the mean particle size was 102±7.95 nm and the zeta potential and polydispersity indices were not measured. The researchers adopted egg yolk lecithin as a lipid and only trichloromethane as a solvent (Liu et al., 2011). In another study where liposomal ceftiofur was produced, the encapsulation efficiency was 39.5% ±1.1%. They reported that no measurement of particle size, zeta potential and polydispersity index was performed (Vilos et al., 2014).

In a review published by Lasic (1998); it was stated that although it was assumed that the encapsulation efficiency in liposomes should be above 70%, this value could not usually be reached experimentally. The encapsulation efficiency, which is around 50%, has been accepted as a high result and it has been found that the efficiencies in the studies is generally around 20-35% (Lasic, 1998). The results of our study showed that it was higher than stated here.

In a study that produced liposomal gentamicin; particle size 408±28-418±21 nm, polydispersity index 0.59±0.009-0.74±0.007, encapsulation efficiency 26.7±1.3-34.5±1.5 µg/µmol. A zeta potential analysis was not performed (Mugabe et al., 2005). Compared to our study, the polydispersity indices were similar. They found the particle size smaller. It is believed that this is due to the difference in the lipid: cholesterol ratio used and the method of chosen.

CONCLUSION

The studies on liposomes continue to increase in many areas. The research and clinical applications are mainly focused on human medicine. However, disease factor resistance is a major problem in veterinary medicine. Effective treatment of disease is important to animal health. This condition ensures healthy consumption of products derived from food-grade animals. Moreover, it play a role in the successful treatment of infections that may a break out in pet animals as well.

Quality control studies should be performed prior to the use of the prepared liposomal formulations in animals. In this study, the quality control studies were performed for liposomal gentamicin and ceftiofur.

According to the results obtained from the study, gentamicin and ceftiofur were successfully obtained in liposomal formulation. It was concluded that pharmacokinetic/pharmacodynamic studies should be performed in animals to evaluate their efficacy.

Acknowledgement

We thank the Drugs and Pharmaceutical Technology Application and Research Center of Karadeniz Teknik University and the Balıkesir University Science and Technology Application and Research Center for their support in conducting the study.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

Plan, design: HS, MC, CC, OC, HS, IK; **Material, methods and data collection:** HS, MC, CC, OC, HC, IK; **Data analysis and comments:** HS, MC, CC, OC, HS, IK; **Writing and corrections:** HS, MC, CC, OC, HS, IK.

Funding

This research was supported by Balıkesir University Scientific Research Projects Unit (BAP2020/095).

REFERENCES

Al-Darraji, A., Donahue, R., Tripathi, H., Peng, H., Levitan, B., Chelvarajan, L., Abdel-Latif, A. (2020). Liposomal delivery of azithromycin enhances its immunotherapeutic efficacy and reduces toxicity in myocardial infarction. *Scientific Reports, 10*. https://doi.org/0.1038/s41598-020-73593-0.

Ali, B. H., Al Za'abi, M., Blunden, G., & Nemmar, A. (2011). Experimental gentamicin nephrotoxicity and agents that modify it: a mini-review of recent research. *Basic & Clinical Pharmacology & Toxicology, 109*(4), 225-232. https://doi.org/10.1111/j.17427843.2011.00728.x.

Bangham, A. D., Standish, M. M., & Watkins, J. C. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology, 13*(1), 238-IN227. https://doi.org/10.1016/S0022-2836(65)80093-6.

Beltrán-Gracia, E., López-Camacho, A., Higuera-Ciapara, I., Velázquez-Fernández, J. B., & Vallejo-Cardona, A. A. (2019). Nanomedicine review: clinical developments in liposomal applications. *Cancer Nanotechnology, 10*(1), 11. https://doi.org/10.1186/s12645-019-0055-y.

Cavanagh, K. (2012). Plumb's Veterinary Drug Handbook. Pocket-7th edition. *The Canadian Veterinary Journal, 53*(12), 1284.

Chou, C. K., Li, Y. C., Chen, S. M., Shih, Y. M., & Lee, J. A. (2015). Chitosan prevents gentamicin-induced nephrotoxicity via a carbonyl stress-dependent pathway. *BioMed Research International, 2015*, 675714. https://doi.org/10.1155/2015/675714.

Çoban, Ö., Barut, B., Yalçın, C. Ö., Özel, A., & Bıyıklıoğlu, Z. (2020). Development and in vitro evaluation of BSA-coated liposomes containing Zn (II) phthalocyanine-containing ferrocene groups for photodynamic therapy of lung cancer. *Journal of Organometallic Chemistry, 925*, 121469. https://doi.org/10.1016/j.jorganchem.2020.121469.

Çoban, Ö., Yıldırım, S., & Bakır, T. (2021). Alpha-lipoic acid and cyanocobalamin co-loaded nanoemulsions: development, characterization, and evaluation of stability. *Journal of Pharmaceutical Innovation*. https://doi.org/10.1007/s12247-020-09531-4.

Gonzalez Gomez, A., Syed, S., Marshall, K., & Hosseinidoust, Z. (2019). Liposomal nanovesicles for efficient encapsulation of staphylococcal antibiotics. *ACS Omega, 4*(6), 10866-10876. doi:10.1021/acsomega.9b00825.

Grazia Calvagno, M. C., Christian; Paolino, Donatella; Cosco, Donato; Iannone, Michelangelo; Castelli, Francesco; Doldo, Patrizia; Fresta, Massimo;. (2007). Effects of lipid composition and preparation conditions on physical-chemical properties, technological parameters and in vitro biological activity of gemcitabine-loaded liposomes. *Current Drug Delivery, 4(1)*, 89-101(113).

Gupta, A., Mumtaz, S., Li, C.-H., Hussain, I., & Rotello, V. M. (2019). Combatting antibiotic-resistant bacteria using nanomaterials. *Chemical Society Reviews, 48*(2), 415-427. https://doi.org/10.1039/C7CS00748E.

Hornish, R. E., & Kotarski, S. F. (2002). Cephalosporins in veterinary medicine - ceftiofur use in food animals. *Current Topics in Medicinal Chemistry, 2*(7), 717-731. https://doi.org/10.2174/1568026023393679.

Kayaalp, O. (2018). Aminoglikozidler. In O. Kayaalp (Ed.), *Akılcıl Tedavi Yönünden Tıbbi Farmakoloji* (pp. 233–241). Ankara: Pelikan Yayıncılık.

Kim, E. M., & Jeong, H. J. (2021). Liposomes: biomedical applications. *Chonnam Medical J., 57*(1), 27-35. https://doi.org/10.4068/cmj.2021.57.1.27.

Lasic, D. D. (1998). Novel applications of liposomes. *Trends in Biotechnology, 16*(7), 307-321. doi:10.1016/s0167-7799(98)01220-7.

Liu, S., Guo, D., Guo, Y., & Zhou, W. (2011). Preparation and pharmacokinetics of ceftiofur sodium liposomes in cows. *Journal of Veterinary Pharmacology and Therapeutics, 34*(1), 35-41. https://doi.org/10.1111/j.1365-2885.2010.01190.x.

Mugabe, C., Azghani, A. O., & Omri, A. (2005). Liposome-mediated gentamicin delivery: development and activity against resistant strains of Pseudomonas aeruginosa isolated from cystic fibrosis patients. *Journal of Antimicrobial Chemotherapy, 55*(2), 269-271. doi:10.1093/jac/ dkh518.

Pattni, B. S., Chupin, V. V., & Torchilin, V. P. (2015). New developments in liposomal drug delivery. *Chemical Reviews, 115*(19), 10938-10966. https://doi.org/10.1021/acs.chemrev.5b00046.

Saddiqi, M. E., Kadir, A. A., Abdullah, F. F. J., Zakaria, M. Z. A. B., & Banke, I. S. (2022). Preparation, characterization and in vitro cytotoxicity evaluation of free and liposome-encapsulated tylosin. *OpenNano*, 100108. https://doi.org/10.1016/ j.onano.2022.100108.

Shen, S., Wu, Y., Liu, Y., & Wu, D. (2017). High drug-loading nanomedicines: progress, current status, and prospects. *International Journal of Nanomedicine, 12*, 4085-4109. doi:10.2147/ijn.S132780.

Vilos, C., Constandil, L., Rodas, P. I., Cantin, M., Zepeda, K., Herrera, N., & Velasquez, L. A. (2014). Evaluation of ceftiofur-PHBV microparticles in rats. *Drug Design, Development and Therapy, 8*, 651-666. https://doi.org/10.2147/dddt.S60444.

Wagner, K., Vorauer-Uhl, K., & Katinger, H. (2006). Nebulization of liposomal rhcu/zn- Sod with a novel vibrating membrane nebulizer. *Journal of Liposome Research, 16*, 113–125. https://doi.org/10.1080/08982100600 680733.