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**ORIGINAL ARTICLE / ÖZGÜN MAKALE**

# PREPARATION AND IN VITRO CHARACTERIZATION OF VANCOMYCIN LOADED PLGA NANOPARTICLES FOR THE TREATMENT OF ENTEROCOCCUS FAECALIS INFECTIONS

*ENTEROKOK ENFEKSİYONLARINDA KULLANILMAK ÜZERE VANCOMİSİN İÇEREN PLGA NANOPARTİKÜLLERİN HAZIRLANMASI VE KARAKTERİZASYONU*

**Gizem Rüya TOPAL[1*], Merve Eylül KIYMACI[2], Yalçın ÖZKAN[3]**

[1]University of Health Sciences, Gulhane Faculty of Pharmacy, Department of Pharmaceutical Biotechnology, 06018, Ankara, Turkey

[2]University of Health Sciences, Gulhane Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 06018, Ankara,Turkey

[3]University of Health Sciences, Gulhane Faculty of Pharmacy, Department of Pharmaceutical Technology, 06018, Ankara,Turkey

## ABSTRACT

**Objective:** *In this study, it was aimed to prepare Vancomycin loaded poly(lactic acid-co-glycolic acid) (PLGA) nanoparticles that can be used parenterally in enterococcal infections.*

**Material and Method:** *In the study, different concentrations of PLGA were used to prepare nanoparticles. Emulsification-solvent evaporation method was used in the preparation of the particles. Particle size and distribution, zeta potential, encapsulation efficiency and yield of the prepared formulations were determined. FTIR analyses were also performed for the formulations. The antibacterial activity was determined as a minimum inhibition concentration by broth microdilution method and as an inhibition zone by disk diffusion method against Enterococcus faecalis ATCC 29212 according to European Committee on Antimicrobial Susceptibility Testing and its effectiveness on bacteria was interpreted by comparing with the pure active substance.*

**Result and Discussion:** *Particles were successfully prepared by emulsion-solvent evaporation method. Around 300 nm; Particles with a high amount of encapsulated Vancomycin were obtained at PDI values of 0.26-0.28. As a result of the FTIR analysis, it was determined that Vancomycin was loaded into the particle. The MIC values of the prepared PLGA nanoparticles against Enterococcus faecalis ATCC 29212 strain was found to be 4 µg/ml, and the inhibition zone diameters were measured as 15 mm, 15 mm and 16 mm, respectively.*

**Keywords:** *Antibacterial activity, Enterococcus faecalis, Nanoparticles, PLGA, Vancomycin*

* **Corresponding Author / Sorumlu Yazar:** Gizem Rüya TOPAL
**e-mail / e-posta:** gizemruya.topal@sbu.edu.tr, **Phone / Tel.:** +03123046050

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## ÖZ

**Amaç:** *Bu çalışmada enterokok enfeksiyonlarında parenteral olarak kullanılabilecek Vankomisin yüklü poli(laktik asit-ko-glikolik asit) (PLGA) nanopartikülleri hazırlanması amaçlanmıştır.*

**Gereç ve Yöntem:** *Çalışmada, nanopartikülleri hazırlamak amacıyla farklı konsantrasyonlarda PLGA kullanılmıştır. Partiküllerin hazırlanmasında emülsiyon oluşturma-çözücü buharlaştırma yöntemi kullanılmıştır. Hazırlanan formülasyonların partikül büyüklüğü ve dağılımı, zeta potansiyeli, enkapsülasyon etkinliği, üretim verimi ölçülmüştür ve FTIR analizi yapılmıştır. Enterococcus faecalis ATCC 29212 suşu üzerine antibakteriyel aktivite sıvı mikrodilüsyon yöntemi ile minimum inhibisyon konsantrasyonu olarak ve disk difüzyon yöntemi ile inhibisyon zonu olarak Avrupa Antimikrobiyal Duyarlılık Testi Komitesi standartlarına göre belirlenmiş, sonuçlar saf etken madde ile karşılaştırılarak yorumlanmıştır.*

**Sonuç ve Tartışma:** *Partiküller emülsiyon-çözücü buharlaştırma yöntemi ile başarılı bir şekilde hazırlanmıştır. Yaklaşık 300 nm civarında; 0.26-0.28 PDI değerlerinde, enkapsüle olan Vankomisin miktarı oldukça yüksek partiküller elde edilmiştir. FTIR ve DSC analizi sonucu vankomisinin partikül içine yüklendiği tespit edilmiştir. Hazırlanan PLGA nanopartiküllerin Enterococcus faecalis ATCC 29212 suşu üzerine MİK değerleri 4 µg/ml bulunmuş, inhibisyon zon çapları ise sırasıyla 15 mm, 15 mm ve 16 mm olarak ölçülmüştür.*


**Anahtar Kelimeler:** *Antibakteriyel aktivite, Enterococcus faecalis, Nanopartikül, PLGA, Vankomisin*

## INTRODUCTION

Enterococci are gram-positive cocci that take shape as singles, pairs, and short chains. The main medium of these bacteria is the gastrointestinal tract of humans and of animals. [1]. They are a common reason for urinary tract infections, bacteremia, and endocarditis and sometimes cause intra-abdominal infections and meningitis [2]. Enterococci have been among the important causes of hospital infections in recent years due to their resistance to environmental conditions, resistance to various antibiotics and their ability to develop new resistance [3]. Vancomycin is a glycopeptide antibiotic that is frequently used in the treatment of enterococci. Long-term and high-dosage regime causes the formation of resistant strains against Vancomycin and the patient is highly affected by side effects. It has been predicted that the use of nanoparticles with low Vancomycin concentration can be a solution to these problems in order to both reduce these side effects caused by Vancomycin and to prevent antibiotic resistance.

Nanoparticles are solid colloidal drug carrier systems prepared from natural or synthetic polymers, ceramic or inorganic elements, ranging in size from 1-1000 nm [4]. The therapeutically used polymeric nanoparticles contain synthetic biodegradable materials that can be converted into biocompatible polymers in the body and reabsorbed naturally [5]. The small size of the nanoparticles and the ability to be synthesized in the desired size are the main reason for their use as a drug delivery system. Besides, the ability to change the surface charge and modification properties of their surface provide many advantages to nanoparticles. Polymeric nanoparticles can enhance the efficacy of antibiotics in many cases with their brilliant advantages. The increased potency of antibiotics in nanoparticles are generally related to some physicochemical properties of particles like modified surface properties, low drug degradation, and enhanced drug absorption and uptake [6].

PLGA is one of the most used biodegradable polymers. It is approved by Food and Drug Administration (FDA) and European Medicine Agency (EMA) in many drug delivery systems for

human usage [7]. There have been several studies of antimicrobial loaded PLGA nanoparticles representing the improved pharmacological and pharmacokinetic behaviour in comparison to pure antimicrobials, so we planned to use this polymer in our formulation studies [8].

In this study, Vancomycin loaded PLGA nanoparticles were developed for the treatment of enterococcus infections. We aimed to use low concentrations of Vancomycin to reduce side effects, increase patient compliance and overcome the Vancomycin resistance instead of using a high dosage of Vancomycin for systemic effect. We searched the effect of PLGA amount on the formulations in terms of particle size and distribution, zeta potential, encapsulation efficiency. Then FTIR, DSC and drug release studies were also carried out. Finally, antimicrobial efficacy of nanoparticles was determined by minimum inhibition concentration and zone of inhibition studies.

## MATERIAL AND METHOD

### Materials

Vancomycin was kindly gifted from Koçak Pharma (Turkey). PLGA (Resomer® RG 504H), polyvinil alcohol (PVA) and Acetonitrile (ACN) were purchased from Sigma Aldrich (Germany). All other chemicals and reagents were of analytical grade.

### Preparation of Vancomycin loaded PLGA nanoparticles

PLGA nanoparticles were prepared by using emulsion-solvent evaporation method [9]. Briefly, different amounts of PLGA were dissolved in ACN. Vancomycin was added to PLGA solution directly and sonicated by probe sonicator (Sonopuls, Bandelin, Germany) for 30 s at 30% amplitude. The organic phase and aqueous phase (1% w/v PVA) were mixed by using homogeniser (IKA T-25 Ultra Turrax, Germany) at 6000 rpm for 5 min. This emulsion was magnetically stirred (Multistirrer15, Velp Scientific, Italy) at 400 rpm to evaporate ACN for over a night. Nanoparticles (NPs) were separated from the aqueous phase by centrifugation at 9000 rpm for 20 min (IEC Centra MP4R, USA) then washed with distilled water and freeze-dried (Christ, Gamma 2–20, USA). The composition of prepared nanoparticles is given in Table 1.

**Table 1.** Composition of Vancomycin loaded PLGA nanoparticles

| Formulation Code | Amount of PLGA (mg) | Volume of ACN (mL) | Volume of %1 PVA (w/v) solution | Amount of Vancomycin (mg) |
|---|---|---|---|---|
| **F1** | 60 | 2 | 10 | 10 |
| **F2** | 80 | 2 | 10 | 10 |
| **F3** | 100 | 2 | 10 | 10 |

## Characterization of nanoparticles

### Particle size and distribution

The particle size and polydispersity index (PDI) of nanoparticles were measured by photon correlation spectroscopy (PCS) (Nicomp Z3000, USA). The nanoparticle suspension was diluted at 1:200 with distilled water. Each sample was measured in triplicate [9].

### Zeta potential

The zeta potential of nanoparticles was detected by laser doppler velocimetry (Nicomp Z3000, USA). The nanoparticle suspension was diluted at 1:50 with distilled water. Each sample was measured in triplicate [10].

### Drug loading (DL) and encapsulation efficiency (EE)

The drug entrapped in the nanoparticles was determined by indirect method from the supernatant. Nanoparticles were separated from the aqueous phase by centrifugation at 9000 rpm for 20 min (Christ, Gamma 2–20, USA) then the amount of Vancomycin in supernatant was analyzed by UV spectroscopy in triplicate. The determination method of Vancomycin was validated according to International Council of Harmanization (ICH) Q2(R1) [11]. Linearity, accuracy, recovery, precision, specificity, detection, and quantification limits were detected in accordance with guideline. For Vancomycin, concentration range between 3.45 and 240 μg/mL the determination coefficient was found 0.999 for water and 0.9996 for PBS pH 7.4 which are really a high value. Detection and quantification limits were found 0.16 and 2.89 μg/mL for water and 1.91 and 4.64 μg/mL for PBS pH 7.4 respectively. For validation parameters, the relative standard deviation (RSD) values were found less than 2%. For specificity, all expicients that was used in formulation were checked at 280 nm and it was found that there is no spectrum. In the light of all these results, it can be said that the analytical method used for Vancomycin was validated and sensitive.

During the calculation of encapsulation and drug loading efficiency, the equations were used in below Equation 1 (Eq.1) and Equation 2 (Eq.2) respectively [12].

$$EE\ of\ Vancomycin\ (\%) = \frac{Amount\ of\ total\ drug - Amount\ of\ unloaded\ drug}{Amount\ of\ total\ drug} \times 100 \quad \text{(Eq. 1)}$$

$$Drug\ loading\ efficiency\ (\%) = \frac{Amount\ of\ loaded\ drug}{Amount\ of\ total\ drug} \times 100 \quad \text{(Eq. 2)}$$

### Production yield

Nanoparticles recovered after preparation were weighed and the production yield (%) was calculated according to Equation 3 (Eq.3) [13, 14].

$$\text{Production yield (\%)} = \frac{Total\ nanoparticle\ amount\ after\ freeze\ drying\ (mg)}{Total\ solid\ material\ amount\ (vankomycin+polymer)(mg)} \times 100 \quad \text{(Eq. 3)}$$

**Drug release study**

In vitro release of Vancomycin from the nanoparticles was evaluated by using the dialysis membrane method. Vancomycin release from the nanoparticles was evaluated in pH 7.4 phosphate buffer saline (PBS) at 37°C on a shaker at 50 rpm (Nuve, Turkey). Nanoparticles were placed in dialysis bags (12–14 kDa, Spectrum Labs, USA) and placed in 50 mL of PBS. At determined intervals, 2 mL samples were taken and the same amount of fresh buffer was added and the amount of Vancomycin was analyzed using UV spectroscopy at 280 nm.

Release kinetics were also evaluated using KinetDSTM software. Zero-order, first-order Hixson Crowell and Higuchi kinetics were applied and $r^2$ values were compared in order to determine the mechanism of drug release [15]. Moreover, the Korsmeyer–Peppas model has applied drug release behaviour analysis of nanoparticles to discriminate release mechanisms from polymeric systems [15, 16].

**Fourier transform infra red spectroscopy (FTIR)**

FTIR spectrums of Vancomycin, PLGA and the formulations were obtained by Spectrum400 spectrometer (Perkin Elmer, USA) to detect the chemical composition of materials. The spectra were recorded in the IR range from 600 to 4000 $cm^{-1}$.

**Differential Scanning Calorimetry (DSC)**

DSC (Perkin Elmer, USA) analysis was carried out at 25°C as initial and 300°C as final temperatures with temperature raise of 10°C/min in nitrogen atmosphere. DSC curves were evaluated by using Pyris software and glass transition temperatures (Tg) were determined.

**Antibacterial activity studies**

Antibacterial activity of the Vancomycin loaded PLGA nanoparticles was tested against *Enterococcus faecalis* ATCC 29212. Antibacterial activity test was evaluated by broth microdilution method as a minimal inhibition concentration (MIC) and by disk diffusion method as an inhibition zone according to European Committee on Antimicrobial Susceptibility Testing standards [17]. In disk diffusion test, MHA plates with 4.0 ± 0.5 mm depth level were used. The bacterial suspension was prepared in 0,9% NaCl saline to the density of a 0,5 McFarland turbidity by a densitometer from fresh culture of *E. faecalis* ATCC 29212. A sterile cotton swab was dipped into bacterial suspension and spreaded on the agar surface. Then the Vancomycin loaded PLGA nanoparticle discs were applied to the surface of the inoculated agar plates and incubated at 35 ± 1°C for 18 ± 2 h. After incubation, zone of inhibition around the test disks were measured and interpreted by comparison with the control. In broth microdilution test, a total of 100 µl cation adjusted MHB was added to

the all U-bottom microplate wells. The same volume of Vancomycin loaded PLGA nanoparticle solutions was added to the first well and diluted. Then the 1:100 dilution of *E. faecalis* ATCC 29212 suspension that was prepared in 0,9% NaCl saline from fresh *E. faecalis* ATCC 29212 culture to the density of a 0,5 McFarland turbidity was added to wells. The microplates were incubated at 35 ± 1°C for 18 ± 2 h. After incubation, minimal inhibition concentration was determined as the lowest concentration of a Vancomycin loaded PLGA nanoparticle solutions that inhibited the visible growth of *E. faecalis* ATCC 29212.

### Statistical analysis

All data were expressed as mean±standard deviation. Student's t test was used to compare differences between groups. P values less than 0.05 were considered statistically significant. Each experiments and analyses were performed as 3 replicates.

## RESULT AND DISCUSSION

### Preparation of nanoparticles and particle size and polydispersity index

Vancomycin loaded PLGA nanoparticles were prepared successfully. Nanosized and monodispersed particles with a narrow range could be obtained using the emulsion-solvent evaporation technique. The formulation contains PVA in its outer phase, which is expected to reduce the migration of hydrophilic Vancomycin from the inner phase to the aqueous phase. This migration is undesirable as it reduces encapsulation efficiency. Therefore, PVA was used as a surfactant in the external phase [14, 18].

As it can be seen in Table 2, particle size and PDI grow as PLGA concentration increased from 60 mg to 100 mg slightly. Increasing polymer concentration can result to enhance the viscosity of the organic phase and formation of larger droplets in the emulsion. Besides, it may lead to disperse of drug-polymer solution into the aqueous phase slowly, so it caused to obtain bigger nanoparticles. This situation can be also responsible for the increase in the PI with increasing concentration of PLGA[19, 20]. This effect was also reported in the literature [21-23].

**Table 2.** Results of characterization studies for Vancomycin loaded PLGA nanoparticles (n=3)

| Formulation code | Particle size (nm) ± SD | PDI ± SD | Zeta potential ± SD (mV) | EE ± SD (%) | DL ± SD (%) | Production yield (%) |
|---|---|---|---|---|---|---|
| F1 | 267.3 ±2.66 | 0.238 ± 0.04 | -10.31 ± 0.09 | 98.39 ± 0.01 | 14.22± 0.01 | 73.93 |
| F2 | 301.4 ± 3.6 | 0.268 ± 0.03 | -10.62 ± 0.01 | 98.60 ± 0.01 | 12.25± 0.01 | 40.06 |
| F3 | 312.5 ±1.85 | 0.284 ± 0.07 | -9.82 ± 0.80 | 98.53 ± 0.01 | 8.91± 0.01 | 24.52 |

**Zeta potential of nanoparticles**

Zeta potential is related to the stability of nanoparticles [24]. The high zeta potential of the nanoparticles could decrease the interaction between particles because of the electrostatic repulsion, so the aggregation possibility of nanoparticles could reduce and it results in high stability [20]. Zeta potential of F1, F2 and F3 were found -10.31, -10.62 and -9.82 respectively (Table 2). For all formulations, the electrical charge was negative. It happened because of the ionized terminal carboxylic groups of PLGA that was on the surface of the particles [25]. A similar study carried out by Zakeri in 2013 was found similar zeta potential results for their particles [12].

**Encapsulation efficiency**

The encapsulation efficiencies of nanoparticles were found similar (Table 2). It was expected that EE values would be affected by different PLGA concentrations higher PLGA amount results in higher EE and many studies support this expectation, but in our study, different PLGA concentrations didn't affect the encapsulation efficiency [12, 25]. It was thought that difference in PLGA amount could be seen when the variation between PLGA concentrations was higher.

**Production yield**

The range of production yield for the particles was about 74%-25% (Table 2). According to the results, F1 formulation had the highest yield value and as PLGA concentration increased, yield values decreased. In different studies, researchers found opposite results [13, 14, 26]. Some studies indicated that it could be about the solubility of PLGA in organic solvent [27]. In F1 formulation, PLGA could be solved completely, it increased the yield of production.

**Drug release study**

The percentages of Vancomycin released from PLGA loaded particles are shown in Figure 1. The release study was carried out for 72h, but after 24h, the release profile didn't change for all formulations and free drug. The release of Vancomycin from nanoparticles was comparatively slow after 24h in comparison with free drug. In 6h, the free drug was completely released to the medium. Nearly, 43%, 30% and 25% of Vancomycin was released from particles F1, F2 and F3, respectively in 24h. It was thought that there was a strong interaction between Vancomycin and polymer due to opposite electric charges, so it led to slow the release profile of Vancomycin according to free form [8, 9]. Also, it can be seen from the release graph that when the amount of polymer was higher which also led to bigger particles, the Vancomycin release from particle was slower. The high concentration of PLGA may have increased the interaction with Vancomycin, resulting in the slower release. In the literature, it was explained that higher concentration of the active substance in nanoparticles generally resulted in faster release, and smaller amounts of the drugs in nanoparticles release in a more sustained manner [22]. Our results were compatible with the literature.

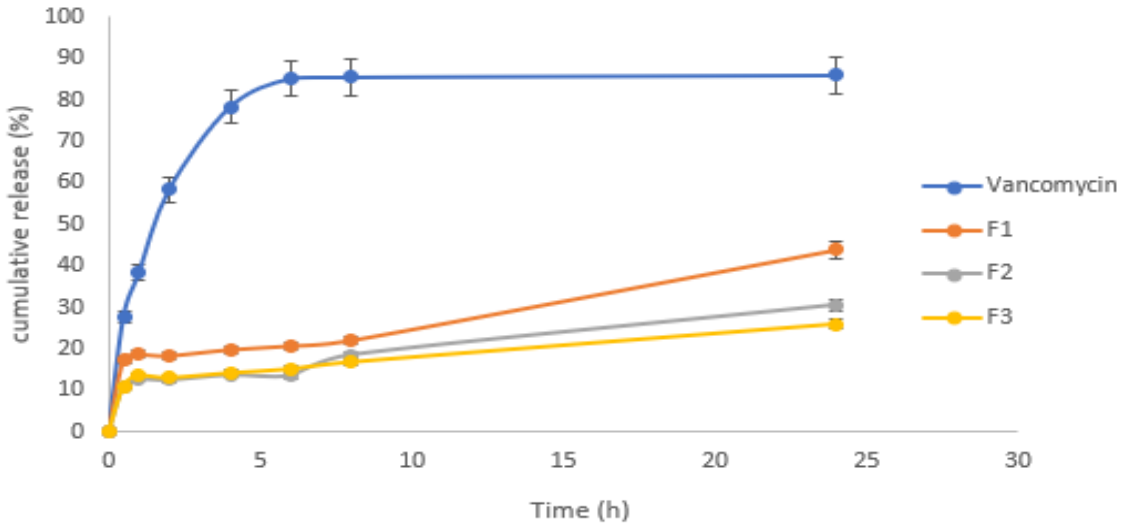


**Figure 1.** Release profile of Vancomycin, F1, F2 and F3

Drug release kinetics were analysed by plotting the cumulative release data to time by fitting to an exponential equation; zero-order kinetics, first-order kinetics, Hixson–Crowell's and Higuchi's equation and the $r^2$ values of these mathematical models were calculated. The $r^2$ values of zero-order kinetics, first order kinetics, Hixson–Crowell's and Higuchi's equation were found 0.9739; 0.9912; 0.9866; 0.1501 for F1; 0.9744; 0.9486; 0.9607; 0.1142 for F2 and 0.9817; 0.9434; 0.9605; 0.1625 for F3 respectively. According to the $r^2$ values, F2 and F3 coded formulations have been fitted with zero order and F3 has been fitted with first-order kinetic. To confirm the drug release mechanism from nanoparticles, the $r^2$ values were calculated by using Korsmeyer and Peppas Equation. According to equation, n is a value that indicating of the release mechanism. For spherical particles, the n values below 0.43 refer to for Fickian diffusion, values between 0.43 and 0.85 refer to anomalous non-Fickian transport, values between 0.85 and 1.00 refer to case II transport and values above 1.00 indicate super case-II transport mechanism [28]. For F1, n and $r^2$ values were found 1.97 and 0.6786; 2.29 and 0.7712 for F2 and 1.87 and 0.8435 for F3 respectively. All the n values for formulations were above 1.00 which was related to super case-II transport. It explains the release mechanism as swelling and polymer chain relaxation-controlled release.

**Fourier transform infra red spectroscopy (FTIR)**

FTIR spectrums of Vancomycin, polymer, and prepared nanoparticles were recorded on Spectrum 400 IR-spectrophotometer. FTIR spectra of Vancomycin showed major characteristics peaks at 3273 $cm^{-1}$ related to COOH, 1650 $cm^{-1}$ related to R-CO-$NH_2$, 1226 $cm^{-1}$ phenolic OH, 1060 $cm^{-1}$ R-O-R. PLGA showed characteristics peaks like C-H stretches at 2950 $cm^{-1}$, C=O at 1753 $cm^{-1}$, C-H bends at 1423 $cm^{-1}$ and C-O stretches at 1168 $cm^{-1}$ [29]. Vancomycin peaks were not seen in the spectra of particles, so the results confirmed that Vancomycin was totally dispersed in polymeric structure (Figure 2).

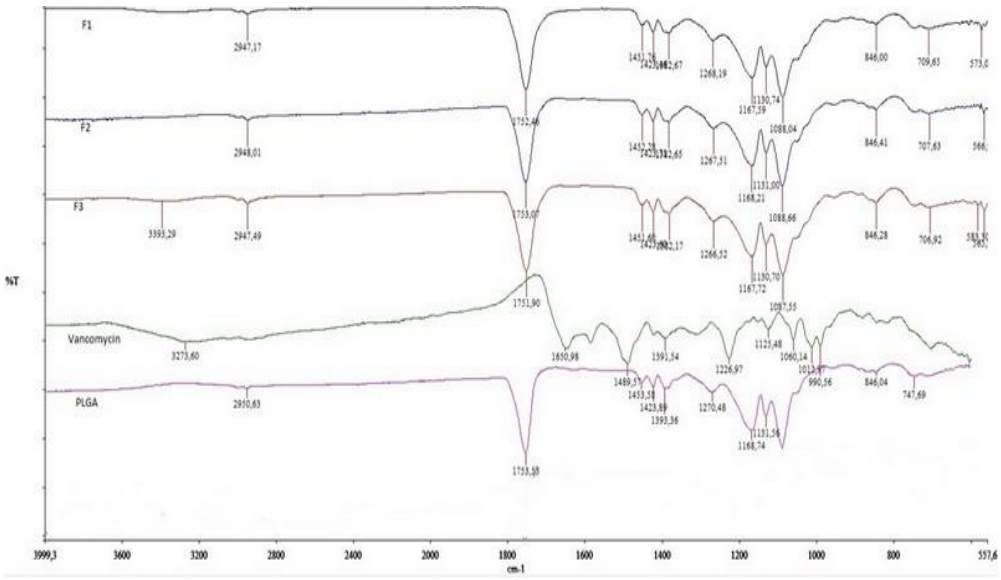


**Figure 2.** FTIR spectrums of formulations, Vancomycin, and PLGA

### Differential Scanning Calorimetry (DSC)

When the DSC thermograms of Vancomycin, PLGA and formulations were examined, Vancomycin peak which was at 83.82° wasn't seen on the DSC thermogram of the formulations [30] (Figure 3). The results showed that the Vancomycin was turned to amorph form and be trapped in polymer matrix of formulation and homogeneously dispersed. The observed peak at formulations belongs to PLGA according to the pure PLGA results. The reason for the appearance of these peaks is that PLGA generates a layer covering the surface of the nanoparticle.

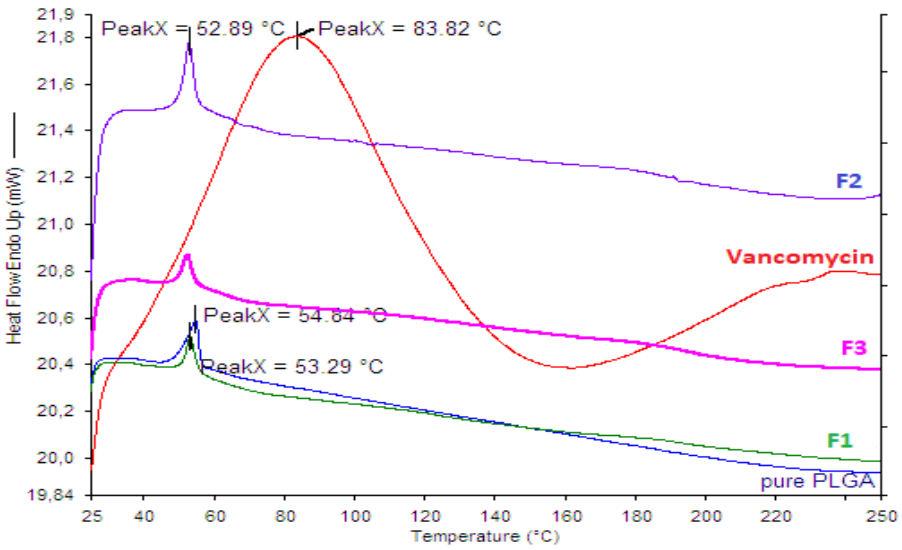


**Figure 3.** DSC thermogram of formulations, Vancomycin, and PLGA

**Antimicrobial Efficacy Studies**

Antibacterial activity of Vancomycin loaded PLGA NPs (60, 80, 100) were investigated by broth microdilution and disk diffusion method. According to same MIC results for all formulations and pure Vancomycin, it was determined that Vancomycin loaded PLGA NPs (60, 80, 90) did not show activity at lower concentrations compared with the pure active substance Vancomycin, so the MIC results did not change. The differences in the inhibition zones obtained from the disk diffusion study for F2 and F3 compared with free Vancomycin were not statistically significant ($p>0.05$), but for F1 the result was significicant ($p<0.05$). The results were shown in Table 3. Vancomycin is a large molecule with a positive charge on its surface. Conversely, PLGA is a hydrophobic drug carrier with a negative charge on its surface, so PLGA and Vancomycin can make a strong interaction because of the electrostatic charge on their surfaces, hence the Vancomycin release from nanoparticles significantly reduces in vitro conditions and also, it can have an influence on antimicrobial effect. There are similar studies that have obtained this result [8, 31, 32]. That can be the reason for F2 and F3 formulations didn't show any changes in comparison with free form. For F1, according to disk diffusion results, there was an enhanced activity compared with free form, so because of this reason F1 can choose for next studies.

**Table 3.** Antibacterial activity results of Vancomycin loaded NPs against *E. faecalis* ATCC 29212.

| Formulation code | MIC (µg/ml) | Zone diameters (mm) |
|---|---|---|
| F1 | 4 | 16 |
| F2 | 4 | 15 |
| F3 | 4 | 15 |
| Vancomycin | 4 | 14 |

In this study, our aim was to develop Vancomycin loaded PLGA nanoparticles to overcome the Vancomycin resistance because of the high and long dosage regime of antibiotics. Three formulations with different PLGA concentrations were prepared and particle size and distribution, zeta potential, encapsulation efficiency, production yield, drug release from particles FTIR and DSC analysis were carried out. The increasing changes of PLGA concentration led increasing particle size and PDI results for the prepared formulations. Zeta potential values were similar. The release of Vancomycin from particles was around 43%, 30% and 25% in 24 hours for F1, F2 and F3 respectively and in comparison with free Vancomycin, release was slower. According to the FTIR and DSC analyses, it can be said that Vancomycin was loaded into PLGA core for all the formulations. According to antimicrobial studies, MIC didn't change significantly compared with pure Vancomycin and inhibition zones of prepared formulations were very similar except F1. The similar antimicrobial effect of PLGA nanoparticles F2, F3 and pure Vancomycin in in-vitro condition is perhaps connected to the strong electrostatic

relationship between hydrophilic Vancomycin and relatively hydrophobic PLGA that results in the slow release of the antibiotic from nanoparticles. Besides, when the PLGA nanoparticles are applied in vivo, increased in vivo stability of Vancomycin and a faster release will be observed. In this case, an increased antimicrobial effect can be expected in-vivo, because of the increased concentration of Vancomycin at in-vivo. For F1, a faster release profile could have resulted in better antimicrobial activity.

The results demonstrated that the antibacterial activity of PLGA nanoparticles can enhance the in vivo activity of Vancomycin, so this formulation study could be a candidate for decreasing the dosage of Vancomycin for further studies.

## AUTHOR CONTRIBUTIONS

Concept: *G.R.T., M.E.K.*; Design: *G.R.T., M.E.K.*; Control: *G.R.T., M.E.K., Y.Ö.*; Sources: *G.R.T., M.E.K., Y.Ö.*; Materials: *G.R.T., M.E.K., Y.Ö.*; Data Collection and/or processing: *G.R.T., M.E.K.*; Analysis and/or interpretation: *G.R.T., M.E.K.*; Literature review: *G.R.T., M.E.K., Y.Ö.*; Manuscript writing: *G.R.T., M.E.K.*; Critical review: *G.R.T., M.E.K., Y.Ö.;* Other: -

## CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

## ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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