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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT OF AMPHOTERICIN-B LOADED NANOPARTICLES AND EVALUATION THE ANTIMICROBIAL POTENCY

AMFOTERİSİN-B ENKAPSÜLE EDİLMİŞ NANOPARTİKÜLLERİN ANTİMİKROBİYAL POTENSİNİN DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: *The aim of this study is the development of Amphotericin B loaded polymeric nanoparticles and the determination of the potency of Amphotericin B nanoformulation samples and commercially supplied Amphotericin B samples in comparison with reference Amphotericin B standard, according to the protocol detailed in the United States Pharmacopoeia.*

Material and Method: *Amphotericin B nanoparticles were fabricated using single emulsion method. The comparison of the potencies of the AmB nanoformulation and commercial Amphotericin B with the antimicrobial potency of the reference Amphotericin B standard was performed using the disk diffusion method specified in the United States Pharmacopeia.*

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Result and Discussion: *Amphotericin B loaded poly(ethylene glycol)-b-poly(ε-caprolactone) nanoparticles successfully developed having the average hydrodynamic diameter of 215.14±0.72 nm and PDI value of 0.18±0.02. The Amphotericin B encapsulation efficiency, which was determined using an HPLC method, was 66.4±1.42%. The % potency of commercial Amphotericin B was calculated as 95.7%, while the % potency of the nanoformulation of Amphotericin B was calculated as 99.1%, indicating the favor of utilizing polymeric nanoparticles as delivery systems.*
Keywords: *Amphotericin B, antimicrobial potency, pharmacopoeia method, polymeric nanoparticles*

ÖZ

Amaç: *Bu çalışmanın amacı, Amfoterisin B yüklü polimerik nanopartiküllerin geliştirilmesi ve Amfoterisin B nanoformülasyon örneklerinin ve ticari olarak temin edilen Amfoterisin B örneklerinin potensinin, Amerika Birleşik Devletleri Farmakopesi'nde ayrıntılı olarak açıklanan protokole göre referans Amfoterisin B standardı ile karşılaştırmalı olarak belirlenmesidir.*
Gereç ve Yöntem: *Amfoterisin B nanopartikülleri tek emülsiyon yöntemi kullanılarak üretilmiştir. Referans Amfoterisin B standardının antimikrobiyal potensi ile ticari Amfoterisin B ve Amfoterisin B'nin nanoformülasyonunun potenslerinin kıyaslanması, Amerika Birleşik Devletleri Farmakopesinde belirtilen disk difüzyon yöntemi kullanılarak gerçekleştirilmiştir.*
Sonuç ve Tartışma: *Ortalama hidrodinamik çapı 215.14±0.72 nm ve PDI değeri 0.18±0.02 olan Amfoterisin B yüklü poli(etilen glikol)-b-poli(ε-kaprolakton) nanopartikülleri başarıyla geliştirilmiştir. HPLC yöntemi kullanılarak belirlenen Amfoterisin B enkapsülasyon etkinliği %66.4±1.42 olarak bulunmuştur. Ticari Amfoterisin B'nin % potensi %95.7 olarak hesaplanırken, Amfoterisin B'nin nanoformülasyonunun % potensi %99.1 olarak hesaplanmış olup bu bulgu, taşıyıcı sistem olarak polimerik nanopartiküllerin kullanılmasının avantajını ortaya koymaktadır.*
Anahtar Kelimeler: *Amfoterisin B, antimikrobiyal potens, farmakope yöntemi, polimerik nanopartiküller*

INTRODUCTION

Polymeric nanoparticles, which are comprised of synthetic or natural polymers, are one of the most frequently utilized forms of nanoparticles for drug delivery. Polymeric based particles have the ability to encapsulate medicinal molecules within their core or matrix, or to attach them to their surface. In order to enhance their interaction with biological systems, polymeric nanoparticles may be made with a variety of forms, sizes, charges, and surface features [1]. Polymeric nanoparticles can also be modified with functional groups or ligands to improve their targeting, controlled release, or stimulus sensitivity. Polymeric nanoparticles, for example, can be coated with antibodies, peptides [2], or aptamers capable of recognizing particular receptors on the surface of target cells or tissues [3]. Polymeric nanoparticles can also be programmed to release medications in response to pH, enzymes, temperature, light, or magnetic field changes. Polymeric nanoparticles for drug molecules encapsulation have many advantages over conventional drug delivery systems. They can increase the loading capacity and stability of drugs, reduce their toxicity and side effects, prolong their circulation time and half-life, and improve their pharmacokinetics and pharmacodynamics. Polymeric micro and nanoparticles for drug molecules encapsulation have been used for delivering various types of drugs, such as anticancer agents [4], anti-inflammatory agents [5], antibiotics, antiviral agents, vaccines, gene therapy vectors, and imaging contrast agents [6]. Polymeric nanoparticles for drug molecule encapsulation have shown promising results in preclinical and clinical studies for treating various diseases and disorders [7].

Amphotericin B (AmB) is an antifungal drug used at antifungal treatments. Coccidioidomycosis, aspergillosis, mucormycosis, candidiasis, and blastomycosis are all significant, life-threatening fungal diseases that can be treated with it. It's also efficient against protozoan parasites like leishmaniasis. AmB acts by binding to ergosterol, a sterol presents in fungal and certain bacteria cell membranes. This impairs the integrity and function of the cell membrane, resulting in cellular content leakage and cell death [8]. Because of its low oral absorption, AmB is commonly taken intravenously. It does, however, have a number of adverse effects, including fever, chills, headache, nausea, vomiting, renal damage, and low blood potassium. As a result, it is reserved for severe or potentially fatal infections that do not

respond to other antifungal medications. AmB is also available in a liposomal formulation, which decreases toxicity and enhances absorption [9]. AmB is a powerful antibacterial drug with a broad spectrum of action that may be used to treat a variety of fungal and parasite illnesses. It does, however, have substantial side effects that necessitate careful monitoring and dose modification. Because of this reason we aimed to formulate AmB with polymeric nanoparticles.

Antimicrobial potency determination is critical for quality control and assurance of antibiotic formulations. Therefore, it is necessary to choose practical and economical methods for quality control of antibiotics. Potency determination can be determined by chemical and biological methods such as microbiological, chemical and immunological tests. The measurement of antibiotic components is usually done by chemical methods including UV and HPLC based analyses. However, these methods cannot reflect real biological activity. Compared to chemical methods, microbiological testing allows to measure the actual effects of antibiotics on biological systems. Microbiological methods are accepted as the standard method because they can reveal small changes in antimicrobial activity that cannot be determined by chemical methods [10].

Determination of antibiotic potency by the microbiological method is a method in which varying concentrations of antibiotics are tested against a living microorganism. Factors such as test microorganism, incubation conditions, amount of inoculum, dose of antibiotic to be tested, preparation of antibiotic standards and equipment are influential on test results. Antibiotic potency determination by microbiological method can be done by plate (cylinder-plate or diffusion) or tube (turbidimetric) methods. While inhibition zones are observed and measured during plate method, turbidity is determined during tube method. The obtained results are evaluated by tabulating and integrating them into a linear regression curve [11].

The aim of this study is the development of AmB loaded polymeric nanoparticles and also the determination of the potency of AmB nanoformulation samples and commercially supplied AmB samples in comparison with reference AmB standard, by disk diffusion method according to United States Pharmacopoeia 2023 (USP) [12].

MATERIAL AND METHOD

Commercial Amphotericin B was purchased from Cayman Chemical and reference Amphotericin B standard (United States Pharmacopoeia reference standard) was purchased Sigma-Aldrich. DMSO, poly(vinyl alcohol) (PVA), and acetonitrile were supplied from Sigma-Aldrich. Poly(ethylene glycol)$_{5000}$-*b*-poly(ε-caprolactone)$_{15000}$ (PEG-PCL) was synthesized and characterized at Yıldız Technical University.

Preparation of AmB Loaded Nanoparticles

The single emulsion-solvent evaporation technique was employed to prepare nanoparticles [13]. Basically, 0.5 mg of AmB is dissolved in 30 µl of DMSO and this solution is mixed with 0.5 ml of chloroform containing 20 mg of PEG-PCL polymer. This solution is added onto the 2.5 ml of PVA solution (1%) and sonicated (35W) on an ice bath for 15 seconds to obtain an emulsion form. Then, obtained emulsion was injected, quickly without making any bubbles, into the 18 ml of PVA solution stirring (1%) on a magnetic stirrer. After 2.5 hours of injection, nanoparticle dispersion was centrifuged at 30.000 rpm for 1 hour, collected, and washed once again then stored for further use.

Determination of the Size and Zeta Potential of Nanoparticles

The zeta potential and particle size distribution of produced nanoparticles were measured using the dynamic light scattering technique. The nanoparticles were diluted at a ratio of 1:50 using pure water and all measurements were done at room temperature using a Zetasizer Nano ZS (Malvern Instruments). The samples were examined three times. Electrophoretic light scattering was used to detect the zeta potential as well.

Determination of the Encapsulated AmB into Nanoparticles

A modified HPLC technique was used to determine the quantity of AmB that was encapsulated

[14]. Briefly, an HPLC equipment with a DAD detector (wavelength set at 388 nm) and a C18 column (Gemini 1004.6 mm, 3m, Phenomenex) was used to determine the quantity of AmB in the sample. Acetonitrile:Buffer (30:70) system was used as the mobile phase, and the injection volume was 20 l. (Buffer: 0.01M Potassium dihydrogen phosphate solution at pH 3.0). Before analysis, the nanoparticle supernatant (0.5 ml), obtained after centrifugation, was mixed 1:1 with ethanol. The analysis was carried out in triplicate at a flow rate of 1.5 ml/min in a column set to 30°C for 10 minutes. The AmB's encapsulation efficiency was presented as percentage encapsulation efficiency.

Morphology Analyses of Nanoparticles

Transmission Electron Microscopy (TEM, FEI Tecnai G2 Spirit) at 120 kV was used to examine the morphology of nanoparticles. The TEM imaging was done on phosphotungstic acid stained nanoparticles. For 2 minutes, 7 µl of nanoparticle dispersion (1.0 mg/ml) was deposited onto carbon coated grids. For 6 seconds, nanoparticles were stained with a 0.75% (w/v) phosphotungstic acid staining solution at pH 7.4. The extra solutions were removed using filtration paper, and the grids were vacuum dried.

Test Microorganisms

In this study, *Saccharomyces cerevisiae* ATCC 9763 strain was used as a test microorganism. *S. cerevisiae* strain was incubated for 48 hours at 29-31°C in M19 medium prepared according to USP. The potency determination was carried out in accordance with the procedure specified in USP 2023 [12].

Preparation of AmB Samples

Potency determination of AmB samples was measured and evaluated according to the median reference standard (S3) and standard curve values (S1, S2, S4, and S5). The samples concentrations were determined according to the USP 2023 [12]. The median concentrations of standard, commercially supplied, and nanoformulation of AmB samples were determined as 25 µg/ml. The tested concentrations of all AmB samples are given in Table 1.

Table 1. The tested concentrations of Amphotericin B samples

Reference Standart Amphotericin B	**S1** 16 µg/ml	**S2** 20 µg/ml	**S3** 25 µg/ml	**S4** 31.25 µg/ml	**S5** 39.0625 µg/ml
Commercial Amphotericin B		**M2** 20 µg/ml	**M3** 25 µg/ml	**M4** 31.25 µg/ml	
Nanoformulation of Amphotericin B		**F2** 20 µg/ml	**F3** 25 µg/ml	**F4** 31.25 µg/ml	

Determination of Potency

The disk diffusion method was used for the determination of potency. Firstly, 25% transmittance at 580 nm in saline was obtained from *S. cerevisiae* ATCC 9763 cultures incubated in M19 medium at 29-31°C for 48 hours. Appropriate amounts of *S. cerevisiae* suspension were added to M19 media cooled to 45-50°C. Then, the media were poured into petri dishes and allowed to solidify. Meanwhile, the samples prepared at the concentrations indicated in Table 1 were absorbed in blank discs. Then the discs were placed on the media as in Figure 1.

After the discs were placed, the petri dishes were incubated at 29-31°C for 48 hours. After incubation period, inhibition zone diameters were measured and evaluated. The study was carried out in triplicate.

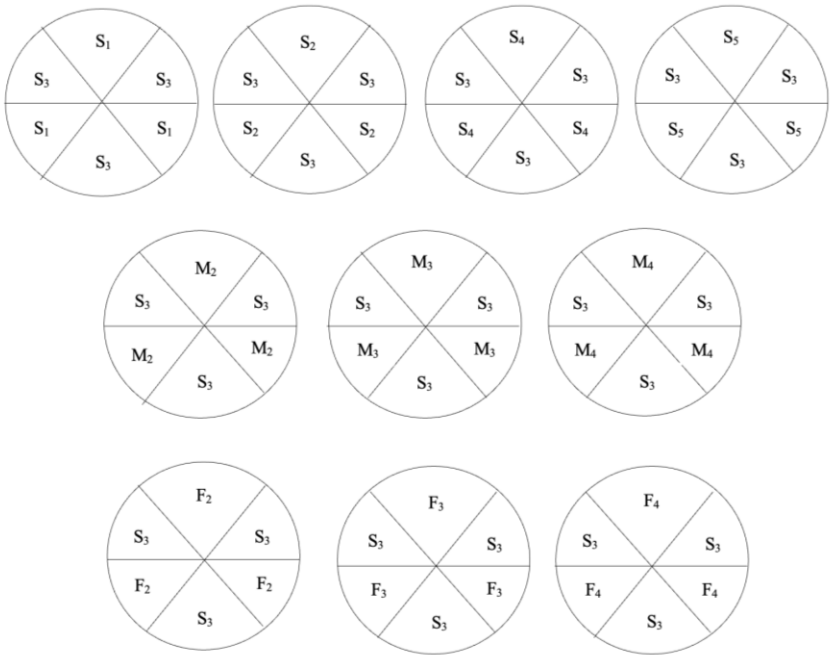

Figure 1. Agar design for determination of AmB potency (S: Reference AmB Standard, M: Commercial AmB, F: Nanoformulation of AmB)

RESULT AND DISCUSSION

AmB loaded nanoformulations were fabricated using single emulsion-solvent evaporation technique which is quite preferred to encapsulate hydrophobic molecules [15]. PEG-PCL copolymer was used to form nanoparticles because of its biodegradation ability and FDA clearance (10.1039/D1CC04941K). Besides, PEG layer limits the undesired interaction with proteins and acts as a steric barrier.

After the nanoformulations were produced, they were subjected to a holistic characterization process. Firstly, the hydrodynamic diameter of the nanoparticles, which indicate their size, were analyzed by dynamic light scattering (DLS) technique. As can be seen from the graph presented in Figure 2A, the average hydrodynamic diameter was determined as 215.14±0.72 nm. The size distribution of the obtained nanoparticles is also in a monomodal narrow distribution as presented in the graph in Figure 2A and the PDI value was found to be 0.18±0.02. In the correlation function graph presented in Figure 2B, the Y-axis intercept value was observed between 0.5-0.9 which indicates that the signal-to-noise ratio in the DLS measurement is at the desired level. The fact that the slope of the same graph is smooth and monomodal (not biphasic and consisting of a single profile) shows that the distribution of the nanoparticles obtained is homogeneous, and the smooth fit with the baseline shows that there are no large aggregates among the nanoparticle population. In addition, the AmB encapsulation efficiency of the nanoparticles was determined as 66.4±1.42% by HPLC analysis and the zeta potential as -17.5±2.3mV which was determined by electrophoretic light scattering method.

After the incubation period of reference standard AmB solution, commercial AmB solution, and AmB naformulation in *Saccharomyces cerevisiae* ATCC 9763, the inhibition zone diameters formed were measured in millimeters. Then mean, standard deviation and RSD% values were calculated. As a result of the study, the R^2 value was calculated as greater than 0.95 and the RSD% value was calculated as less than 10%. The study was conducted at a 95% confidence interval. Inhibition zones formed as a result of the experiment are given in Figure 3.

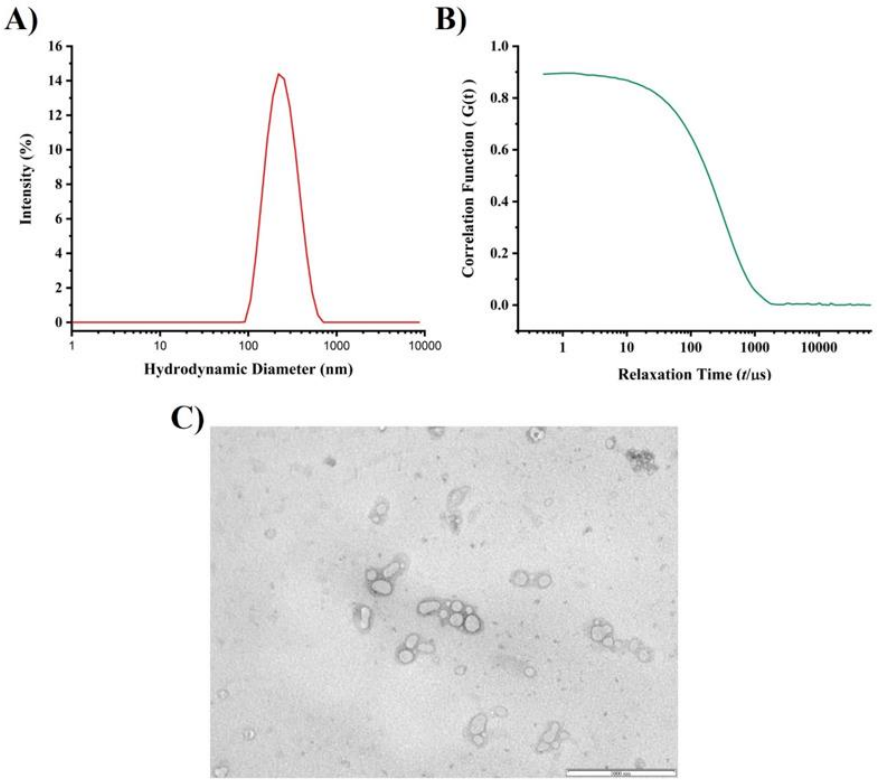

Figure 2. Dynamic light scattering data showing the hydrodynamic diameter (A) and correlation function (B) of the AmB loaded nanoparticles. TEM image of the AmB loaded nanoparticles (C)

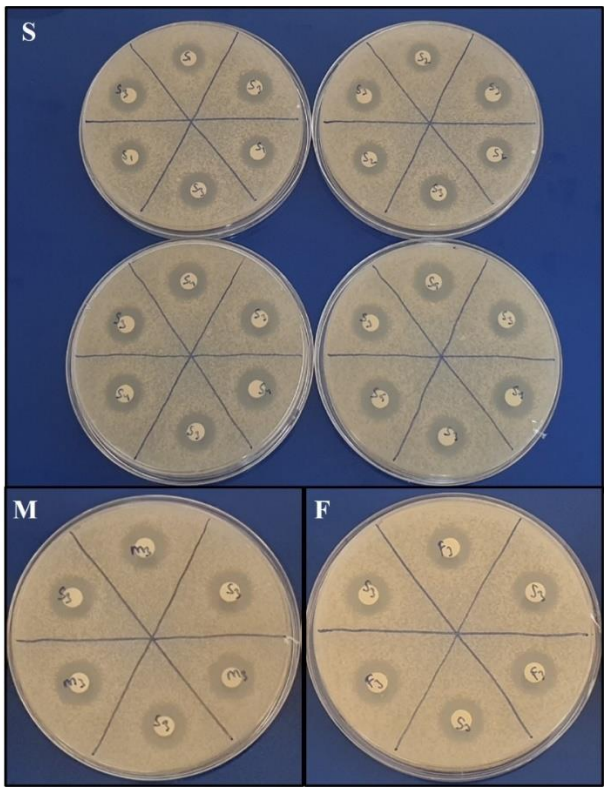

Figure 3. Inhibition zones of reference AmB standard (S), commercial AmB (M), and nanoformulation of AmB (F) samples against *Saccharomyces cerevisiae* ATCC 9763 standard strain

As a result of the experiment, the % potency of commercially supplied AmB was calculated as 95.7%, while the % potency of the nanoformulation of AmB was calculated as 99.1%. These finding indicate the enhanced antimicrobial potency of AmB nanoformulations compared to AmB solution. The utilization of nanoparticles to formulate AmB ending up with synergistic activity might dependend on the increased uptake of of AmB molecules into microbial cell, via nanoparticles [16].

Consequently, AmB loaded PEG-PCL nanoparticle successfully developed and characterized in terms of hydrodynamic diameter, poly dispersity index, zeta potential, and AmB content. The effect of nanoformulation procedure on AmB's antimicrobial potency was unknow while the antimicrobial potency tests detailed in various pharmacopoeias are prerequisite to show formulations efficicacy. Here in this work, we have not only shown that the AmB molecule keeps its antimicrobial potency after the harsh nanoparticle production process, but we also determined that the AmB nanoformulation has a higher antimicrobial effect compared to pristine AmB.

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AUTHOR CONTRIBUTIONS

Concept: U.C.Ö., S.S.R., E.Ş.A., G.C., M.E., A.B.; Design: U.C.Ö., S.S.R., E.Ş.A., H.B., S.D., M.E., A.B.; Control: H.B., S.D.; Sources: E.Ş.A., A.B.; Materials: E.Ş.A., H.B., S.D.; Data Collection and/or Processing: U.C.Ö., S.S.R., G.C.; Analysis and/or Interpretation: U.C.Ö., S.S.R., E.Ş.A., G.C., H.B., S.D., M.E., A.B.; Literature Review: U.C.Ö., S.S.R.; Manuscript Writing: U.C.Ö., S.S.R.; Critical Review: E.Ş.A., H.B., S.D., A.B.; 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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