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Research Article

IN VIVO ASSESSMENT OF POLYLACTIC CO-GLYCOLIC ACID NANOBUBBLES FOR TRIGGERED ENZALUTAMIDE DELIVERY: OPTIMIZATION, CHARACTERIZATION, AND DESIGN OF EXPERIMENTS-BASED EVALUATION

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ABSTRACT

Objectives: This study aimed to develop and optimize polylactic co-glycolic acid (PLGA) nanobubbles (NBs) for the targeted and triggered delivery of enzalutamide (ENZ) using ultrasound-assisted activation. The formulation was designed to enhance drug bioavailability, prolong circulation time, and improve therapeutic efficacy while minimizing systemic side effects.

Methods: ENZ-loaded PLGA NBs were synthesized using the solvent evaporation method, followed by optimization through the Box–Behnken design. The NBs were characterized for particle size (PS), polydispersity index (PdI), zeta potential (ZP), entrapment efficiency (EE), morphology, drug-excipient interactions, thermal stability, and crystallinity. *In vitro* drug release studies were conducted with and without ultrasound exposure. Pharmacokinetic evaluation was performed *in vivo* to assess systemic absorption, bioavailability, and drug retention.

Results: The optimized ENZ-loaded NBs exhibited a PS of 193.5±2.8 nm, PdI of 0.261±0.016, ZP of 31.4±1.17 mV, and EE of 65.12±2.54%. Fourier-transform infrared, differential scanning calorimetry, and X-ray diffraction analyses confirmed successful drug encapsulation without significant chemical interaction. Scanning electronic microscopic analysis revealed uniform, spherical NBs. *In vitro* drug release studies demonstrated significantly enhanced drug release under ultrasound activation due to cavitation-induced NB disruption. Pharmacokinetic studies indicated that drug-loaded NBs achieved a significantly higher C_{max} (1528.06±148.66 ng/mL) and area under the curve 0-∞ (65297.31±546.20 ng·h/mL) compared to the pure drug, with an extended half-life (43.07±3.13 h) and mean residence time (59.35±6.20 h), confirming improved bioavailability and sustained release.

Conclusion: The study successfully formulated and optimized ultrasound-responsive PLGA NBs for ENZ delivery. The *in vivo* findings demonstrated enhanced systemic exposure and prolonged drug release, confirming the potential of NBs as an effective drug delivery system. These results highlight the feasibility of ultrasound-assisted NBs for targeted prostate cancer therapy, warranting further investigation into their clinical applications.

Keywords: Polylactic co-glycolic acid nanobubbles, Enzalutamide, Targeted drug delivery, Ultrasound-triggered release, Pharmacokinetics, Prostate cancer.

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INTRODUCTION

Globally, prostate cancer (PC) is a widespread concern, with around 1.4 million new cases reported in 2020. PC ranks as the 2nd most prevalent form of cancer and stands as the fifth primary contributor to cancer-related fatalities in men across the globe [1]. In India, PC is also a significant health issue, accounting for a significant proportion of cancer cases in men. The occurrence of PC in Asian countries has been increasing in recent years, primarily related with the aging population and changing lifestyles [2]. However, awareness and screening programs in India are still in the early stages of development, highlighting the need for increased efforts in prevention, early detection, and access to high-quality care to alleviate the burden of PC in the country [1].

Androgen deprivation therapy is a well-established treatment strategy for locally advanced and metastatic PC, providing relief from symptoms and producing positive biochemical and objective responses [3]. Before 2012, non-steroidal antiandrogen drugs such as bicalutamide and flutamide were commonly utilized but proved ineffective as the cancer progressed to a hormone-refractory stage [4]. However, since 2012, the introduction of novel second-generation antiandrogen drugs such as enzalutamide (ENZ) has brought about new possibilities in the treatment of castration-resistant PC (CRPC), addressing the limitations of previous therapies [5].

ENZ serves as an androgen receptor signaling inhibitor, effectively halting the proliferation of PC cells, and inducing their apoptosis. By explicitly targeting androgen receptor signaling pathways, ENZ suppresses cancer cell growth and triggers their programmed cell death. This dual action highlights the efficacy of ENZ in treating PC and underscores its potential as a valuable therapeutic tool in combating this disease [6-8]. ENZ received approval from the U.S. Food and Drug Administration in the year 2012 for the treatment of metastatic CRPC, and apalutamide was approved for treating non-metastatic CRPC in 2018 [9,10].

ENZ is classified as biopharmaceutics classification system class II owing to its high permeability and low solubility in water (0.00136 mg/mL), with a log p of 3.75 and melting point of 201°C [9]. The chemical formula is $C_{21}H_{16}F_4N_4O_2S$. The bioavailability of the drug is only 1%. Currently, the drug is available in two strengths (40 and 80 mg/p.o). The drug binds predominantly to plasma proteins, mainly albumin, with a binding rate of 97–98%. Despite its therapeutic potential, the drug exhibits low bioavailability, necessitating high doses for efficacy, which poses a significant limitation.

Different authors have employed various approaches to improve ENZ's solubility by utilizing solid-form optimization techniques. These approaches encompass solid dispersion, nanocrystals, and the integration of polymers such as polyethylene glycol 6000, β-cyclodextrin (β-CD), and modified CDs [11]. Self-nano-emulsifying drug delivery systems (SNEDDS) [12], solid SNEDDS [13], and solid lipid nanoparticles were reported [14].

Despite advancements, achieving deep tissue penetration and effective drug distribution remains a challenge for some of the above approaches. Addressing these limitations through continued research and innovation is crucial to fully realize the therapeutic potential of quercetin in treating various diseases. In addition to improving solubility, it is crucial to have a method that can direct drug molecules to diseased tissues while minimizing their presence in healthy tissues. The targeted delivery system will enhance the concentration of the drug in the blood while improving the pharmacokinetics of the drug while reducing the side effects. Innovative delivery systems, particularly those aimed at cancer treatment, are an example of such systems.

Nanobubbles (NBs) are utilized in various fields, particularly in drug delivery systems, due to their exceptional physical characteristics. They offer remarkable stability, high internal pressure, and a large surface-to-volume ratio [15,16]. Polylactic co-glycolic acid (PLGA), an exceptional nano/microbiomaterial, is extensively utilized in various fields, including targeted drug delivery, molecular diagnostics, tissue engineering, and gene transfer. To the best of our knowledge and based on the available literature, there are no prior studies documenting the utilization of PLGA NBs for delivering ENZ.

Pharmacokinetic studies (PKs) play a crucial role in optimizing drug therapy for PC by evaluating the absorption, distribution, metabolism, and excretion of therapeutic agents. These studies help determine the drug's bioavailability, half-life, and optimal dosing regimen to enhance efficacy while minimizing toxicity. ENZ, a second-generation androgen receptor inhibitor, is widely used in treating mCRPC. However, challenges such as poor solubility, limited bioavailability, and potential resistance necessitate advanced drug delivery approaches. Nanocarrier-based systems, such as PLGA NBs, offer a promising strategy for improving drug pharmacokinetics by enhancing targeted delivery, prolonging circulation time, and enabling triggered release at tumor sites. *In vivo* pharmacokinetic evaluations provide critical insights into these formulations' therapeutic potential, guiding their clinical translation for more effective PC management.

This research fills a critical gap in the field by utilizing PLGA NBs for ENZ delivery and employing the design of experiments (DoE) approach for optimization. DoE streamlines the refinement of experimental variables through systematic strategies and statistical analysis. The study encompasses the design, formulation, and optimization of drug-loaded PLGA NBs, followed by extensive *in vitro* characterization and *in vivo* pharmacokinetic and biodistribution studies. The *in vivo* evaluation focuses on drug release kinetics, systemic circulation time, tumor targeting efficiency, and therapeutic efficacy, providing valuable insights into the potential clinical application of PLGA NBs for PC treatment.

METHODS

Reagents and chemicals

ENZ is a pure drug given by Dr. Reddy's Laboratories, a private limited company in Hyderabad. C3F8 (Perfluoro propane) was procured from pharm affiliates Pvt. Ltd, Haryana, India. Sigma Aldrich, US, supplied Poly (D, L-lactide-co-glycolide) 50:50 with an intrinsic viscosity of 0.22 dL/g and Mw 25,000. Polyvinyl alcohol (PVA; Mw 30,000–70,000) was purchased from Sigma Aldrich (St. Louis, MO, USA). Isopropanol and dichloromethane (DCM) were acquired from S.D. Fine Chemicals, Hyderabad. All other solvents were purchased from Qualigens, India. The Nutrition National Institute (NIN), situated in Telangana, India, provided the male Wistar rats.

Analytical method development using reverse phase- high-performance liquid chromatography (HPLC)

Chromatographic analysis of ENZ was executed using a Shimadzu Prominence model LC-20AD equipped with a ultraviolet detector set at 270 nm for ENZ. A reverse phase Luna C-18 column (150 mm × 4.6 mm i.d., 5.0 µm practical size and 100 Å pore size) maintained at 40.0±0.1°C was employed. The mobile phase is composed using a blend of ammonium acetate buffer (40 mM) and acetonitrile in the ratio of 60:40 (v/v) and the flow rate was adjusted to 1.5 mL/min.

A standard stock solution of the drug at a concentration of 1000 µg/mL was prepared, followed by the creation of various working standard solutions ranging from 0.5 µg/mL to 100 µg/mL through serial dilutions. A stock solution (1000 µg/mL) of nilutamide as internal standard (IS) after appropriate dilution was included in the experiment [17].

ENZ-NBs formulation development and optimization

The study employed a three-factor, three-level Box–Behnken design (BBD) to optimize PLGA NBs loaded with ENZ. The independent variables included stabilizer concentration (% w/v of PVA), homogenization speed (rpm), and homogenization time (min), while the dependent variables (responses) were particle size (PS) (nm), polydispersity index (PdI), zeta potential (ZP; mV), and entrapment efficiency (EE; %). A total of 17 experimental runs, including three center points, were conducted to ensure experimental validity.

ENZ-loaded PLGA NBs were synthesized using a modified solvent evaporation method with ultrasound assistance. PLGA (200 mg) was dissolved in DCM, followed by the addition of ENZ to form a dispersion. This mixture was sonicated at 45% amplitude for 5 min in an ice bath. The dispersion was then emulsified with 25 mL of chilled 1.9% PVA solution and homogenized at 12,200 rpm for 12 min. Subsequent sonication at 30 W for 3 min was performed before adding 25 mL of 2.5% v/v isopropanol, with mechanical stirring for 5 h to remove DCM. The resulting product was centrifuged at 8000 rpm for 5 min, washed with distilled water, and freeze-dried for 36 h under light-protected conditions. Finally, C_3F_8 gas was introduced into the lyophilization chamber at 50 mL/min for 4 min, and the vials were sealed for further analysis [18,19].

Characterization and evaluation

The physicochemical characterization of ENZ-loaded PLGA NBs involved measuring PS, PdI, and ZP using dynamic light scattering with a Zetasizer after tenfold dilution in double-distilled water [19]. EE was determined to assess drug loading capacity, stability, and release kinetics, with samples dissolved in DCM, sonicated, diluted, and analyzed through HPLC [20]. Morphological analysis was performed using scanning electron microscopy (SEM) with Au-sputter coating and imaging at a magnification of 500–10,000× [21]. Fourier-transform infrared (FTIR) spectroscopy examined potential molecular interactions of the drug, polymer, and optimized formulation between 4000 and 450 cm^{-1} [22]. Differential scanning calorimetry (DSC) and X-ray diffraction (XRD) evaluated the drug's physical state, thermal properties, and crystalline nature, with DSC conducted from 50 to 400°C at 5°C/min and XRD recorded at 2°–60° 2θ [13]. Drug release studies were performed using a shake flask-dialysis system in phosphate buffer (pH 7.4) at 37°C, with periodic sampling and HPLC analysis at 270 nm [22]. Stability studies assessed the optimized formulation under different storage conditions (4°C, 25°C, and 40°C at 75% relative humidity), monitoring PS, PdI, ZP, and EE changes over time [23].

PKs

The NIN, situated in Telangana, India, provided the male Wistar rats used in the study, which had an approximate weight of 200±20 g and an age of 4–5 weeks. The animal study followed the guidelines as per the Care and Use of Laboratory Animals. The Institutional Animal Ethics Committee officially sanctioned the protocols designated by the assigned protocol number (1447/PO/Re/S/11/CPCSEA-86/A). Animals were exposed to natural light/dark settings for 1 week, acclimating to a relative humidity of 40–60% and a temperature of 20°C±2°C. After that, they were randomly divided into two groups of six animals. The optimized drug-loaded NBs (40 mg/kg body weight) and the pure drug (dispersed in 0.5% w/v carboxymethylcellulose) were administered by oral route. The animal blood was obtained from the retroorbital plexus (300 µL) and then transferred into sterile test tubes with ethylenediaminetetraacetic acid at specific intervals (0.25,

0.5, 1, 2, 4, 6, 12, 24, 48 and 72 h). Blood samples were centrifuged at 7500 rpm for 10 min using an Eppendorf centrifuge. The resulting plasma was further analyzed using HPLC [12].

The drug was recovered from plasma samples through the protein precipitation method. The drug was successfully extracted from plasma by adding acetonitrile (250 µL) to rat plasma (50 µL) and vortexed. The supernatant was centrifuged for 12 min at 8000 rpm and then analyzed using chromatography at a λmax of 270 nm. Non-compartmental analysis WinNonlin (version 3.1; Pharsight *et al*., USA) was employed to calculate the C_{max} (maximum plasma concentration, (area under the curve [AUC_{0-72}]) area under plasma concentration versus time curve from 0 to 72 h, T_{max} (time to reach the maximum plasma concentration, Kel (elimination rate constant, $t_{½}$ (half-life). All the data were expressed as mean±standard deviation.

Statistical analysis was performed using Design–Expert® software (Version 12.0.3.0, Stat-Ease Inc., USA), and analysis of variance was applied to determine the significance of variables influencing the responses. Regression analysis and response surface methodology were utilized for optimization, and pharmacokinetic parameters (C_{max}, T_{max}, AUC_{0-t}, $AUC_{0-\infty}$, $t_{½}$, Ke) were analyzed with WinNonlin (version 3.1, Pharsight, USA). The desirability function was used to determine the optimal formulation. This comprehensive experimental setup ensured accuracy, reproducibility, and statistical validation of the NB-based drug delivery system.

RESULTS

ENZ-NBs formulation development and optimization

The study aimed to formulate ENZ-NBs by integrating ultrasound technology with solvent evaporation. The drug had been mixed with PLGA that had been dissolved in DCM, sonicated, and added to a cooled 1.9% w/v PVA solution. After that, a 3-min sonication at 30 W and high-speed homogenization were done. Isopropanol solution (2.5% v/v) was used to extract DCM. For extracting, isopropanol was mixed under stirring for 5 h. The word "NBs" was chosen over "nanodroplets" because perfluoropentane is a fluid substance at normal temperature. Acoustic droplet vaporization, a liquid-to-vapor phase change brought on by ultrasound, converted nanodroplets into NBs, enhancing their echogenic qualities in ultrasonography photographs [24].

Characterization and evaluation of NBS

The optimized ENZ-loaded PLGA NBs exhibited a PS of 193.5±2.8 nm, a PdI of 0.261±0.016, and a ZP of 31.4±1.17 mV, ensuring colloidal stability [19]. High ZP values prevent particle aggregation, maintaining NB integrity during storage and administration. FTIR analysis confirmed minor interactions between ENZ and PLGA, suggesting hydrogen bonding without significant chemical modifications [13]. DSC revealed shifts in melting points, indicating drug confinement within the polymeric matrix, while XRD analysis showed the disappearance of ENZ's crystalline peaks, suggesting molecular-level dispersion within NBs [13]. Drug release studies demonstrated significantly enhanced release from NBs compared to pure ENZ, with ultrasound further accelerating release through cavitation-induced NB disruption. The cumulative drug release at 8 h was 16.03±3.32% for plain drug, 44.64±4.61% for NBs without ultrasound, and 78.84±4.04% with ultrasound, reaching over 98.24±8.18% at 48 h under ultrasound conditions [25-28]. Acoustic waves contributed to drug release by triggering NB shell rupture, aligning with previous findings on ultrasound-assisted drug delivery. Stability studies at 4°C, 25°C, and 40°C over 3 months indicated minimal degradation at lower temperatures, with EE slightly decreasing from 65.12±2.54% to 62.41±3.90% at higher temperatures, suggesting potential structural disruptions [28]. The formulation maintained PS below 200 nm and ZP around 29±2.29 mV, ensuring stability. Storage in polyethylene pouches led to a faster drop in concentration compared to glass bottles, highlighting the role of hydrogen bonding in NB stability [28].

PK studies

Fig. 1 displays the plasma concentration-time curve after drug administration in 0.25% w/v sodium carboxymethylcellulose solution and the optimized NBs orally. PK data in Table 1 reveal that the formulation exhibited significantly higher T_{max}, C_{max} (**p<0.001), AUC_{0-24} (**p<0.001), and $AUC_{0-\infty}$ (**p<0.001) values compared to the pure drug suspension at the prescribed dose. The bioanalytical chromatogram indicated drug retention time at 12.7 min and IS (nilutamide) at 9.5 min (Fig. 2).

The optimized formulation reached a maximum level (C_{max}) of 6.84 times higher, while the area under the curve (AUC_{0-t}) was 5.874 times higher than the free drug. *In vivo* studies revealed a progressive drug release from the NB preparation with extended T_{max}. Comparing the data to the free drug, oral bioavailability has significantly improved. This finding suggests a notable improvement in oral bioavailability compared to the free drug. The enhanced bioavailability can be attributed to the increased drug circulation at the nanoscale and the improved penetration facilitated by the polymeric carrier system.

DISCUSSION

This study utilized the solvent evaporation method to formulate ENZ-loaded PLGA NBs, optimizing the process through the BBD. NBs are gaining attention for targeted drug delivery due to their echogenicity and ultrasound responsiveness. Perfluoropentane, used as the inner core, transitions from liquid to vapor upon ultrasound exposure through acoustic droplet vaporization, enhancing ultrasound imaging and drug release efficiency [24,23]. PLGA was chosen for its biocompatibility and biodegradability, making it suitable for medical applications [29]. Ultrasound-induced cavitation and sonoporation effects reduced bubble size and facilitated cellular uptake, improving drug localization while minimizing off-target effects [15]. The quadratic model demonstrated that stabilizer concentration significantly influenced PS, PdI, ZP, and EE, with higher stabilizer levels reducing drug entrapment due to competition for surface adsorption and increased viscosity [30]. FTIR confirmed drug-excipient compatibility, while DSC and XRD indicated drug amorphization within NBs [13]. SEM analysis revealed spherical, smooth-surfaced NBs, and ultrasound-assisted cavitation enhanced drug release through structural disruption. Stability studies

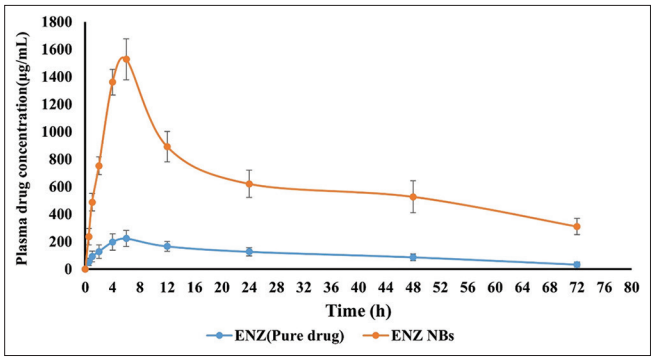

Fig. 1: *In vivo* pharmacokinetic studies

Table 1: Pharmacokinetic parameters

Pharmacokinetic parameters	Pure drug	Drug loaded NBs
C_{max} (ng/mL)	223.2±58.04	1528.06±148.66
T_{max} (h)	6	6
Half-life (h)	26.09±2.58	43.07±3.13
AUC_{0-t} (ng. h/mL)	7833.88±263.16	46022.12±586.82
AUC_{0-inf} (ng. h/mL)	9082.47±420.17	65297.31±546.20
Ke (h^{-1})	0.0265	0.0160
MRT (h)	38.036±5.44	59.348±6.20

NBs: Nanobubbles, AUC: Area under the curve, MRT: Mean residence time

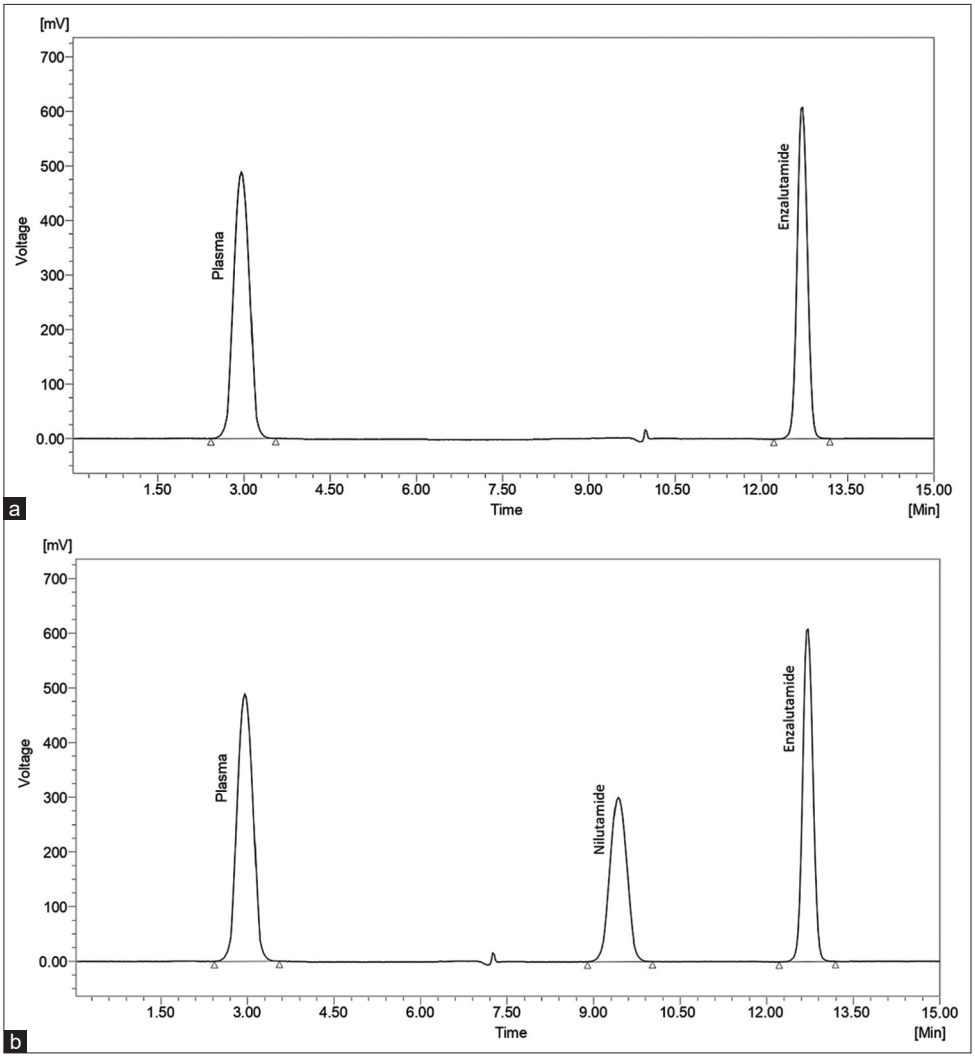

Fig. 2: The bioanalytical chromatogram drug in plasma (a); drug retention in plasma (b) drug and internal standard in plasma indicated drug retention time at 12.7 min and internal standard (nilutamide) at 9.5 min

confirmed acoustic droplet generation, highlighting ultrasound's role in NB-mediated drug delivery [31]. The study aligned with previous findings on NB stability under varying temperature conditions. The temperature-dependent behavior observed in PS, ZP, and entrapment emphasized understanding NB characteristics in diverse environmental conditions for practical drug delivery applications. Different polymer materials submerged in NB dispersions exhibited varied effects on NB number concentrations due to hydrophobic interactions.

The PK analysis highlights the significant improvement in drug absorption, distribution, and retention when encapsulated in PLGA NBs compared to the pure drug formulation. The C_{max} of the drug-loaded NBs (1528.06±148.66 ng/mL) was substantially higher than that of the pure drug (223.2±58.04 ng/mL), indicating enhanced bioavailability. Despite both formulations exhibiting the same time to reach peak concentration (T_{max}) at 6 h, the drug-loaded NBs demonstrated prolonged circulation time, as evidenced by an extended half-life (43.07±3.13 h) compared to the pure drug (26.09±2.58 h). This suggests that the NB formulation reduces drug clearance and enhances systemic retention. The area under the plasma drug concentration-time curve (AUC), a key parameter for evaluating drug exposure, was significantly greater for the NBs. The AUC_{0-t} increased from 7833.88±263.16 ng·h/mL for the pure drug to 46022.12±586.82 ng·h/mL for the NBs, while the $AUC_{0-\infty}$ was also considerably higher (65297.31±546.20 ng·h/mL vs. 9082.47±420.17 ng·h/mL for the pure drug). These findings confirm that NBs significantly enhance drug bioavailability by improving systemic circulation and minimizing rapid elimination. The elimination rate constant (Ke) of the drug-loaded NBs (0.0160 h^{-1}) was lower than that of the pure drug (0.0265 h^{-1}), further indicating a slower elimination process, which supports the prolonged half-life observed. In addition, the mean residence time (MRT) for the NB formulation (59.348±6.20 h) was notably longer than that of the pure drug (38.036±5.44 h), reinforcing the sustained-release capability of the NBs.

Overall, these PK results demonstrate the potential of PLGA NBs in significantly enhancing drug delivery by improving bioavailability, prolonging systemic circulation, and enabling controlled and sustained release. The encapsulation of ENZ in NBs provides a promising strategy for optimizing therapeutic efficacy while minimizing dosing frequency and potential side effects [32].

CONCLUSION

The *in vivo* PK studies demonstrated that PLGA NBs significantly enhanced the systemic bioavailability and retention of ENZ compared to the pure drug formulation. The drug-loaded NBs exhibited a markedly higher C_{max} and AUC, indicating improved drug absorption and prolonged circulation time. The extended half-life and MRT further confirmed the sustained-release capability of the NBs, reducing the frequency of administration and potentially minimizing systemic side effects. The reduction in the elimination rate constant suggests

a controlled release profile, which is beneficial for maintaining therapeutic drug levels over an extended period. These findings highlight the potential of ultrasound-responsive NBs as an effective drug delivery system, offering targeted, non-invasive, and sustained drug release for improved therapeutic outcomes. Future studies should focus on evaluating the biodistribution, therapeutic efficacy, and safety of NBs in relevant disease models to further validate their clinical applicability.

AUTHOR'S CONTRIBUTION

NK contributed to the study design and collaborated with data collecting and analysis, while KM supported in paper writing, assuring a collaborative and balanced effort throughout the research process. Both Authors reviewed and approved the final draft.

CONFLICT OF INTEREST

None.

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