Effect of surfactant types and concentrations on levofloxacin-loaded PLGA microparticles for pulmonary delivery – An in vitro study

Selin İMAMOĞLU[1] , **Burcu DEVRİM GÖKBERK[2]*** , **Müjde ERYILMAZ[3]** , **Asuman BOZKIR[2]**

1   Turkish Medicines and Medical Devices Agency, Ankara, Turkey.
2   Department of Pharmaceutical Technology, Faculty of Pharmacy, Ankara University, Yenimahalle 06560 Ankara, Turkey.
3   Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Ankara University, Yenimahalle 06560 Ankara, Turkey
*   Corresponding Author. E-mail: burcudev@gmail.com (B.D.G.); Tel. +00-312-203 31 62.

ABSTRACT: The aim of this work was to develop levofloxacin-loaded drug delivery systems for pulmonary administration. Levofloxacin-loaded PLGA microparticles were prepared by water-in-oil-in-oil (w/o/o) emulsion solvent evaporation method using PLGA as the polymer. Then, the effect of surfactant type and concentration in the inner aqueous phase or outer oily phase on the physicochemical characteristics of the microparticles were investigated. PLGA-based microparticles have spherical shape with a particle size of about 5 μm, which is suitable for pulmonary delivery. Very high values for encapsulation efficiency (up to 90%) were obtained. The in vitro release of levofloxacin from the PLGA microparticles was sustained for 24 hours. The aerodynamic diameter and fine particle fraction were 5.44 ± 0.19 μm and 50.99 ± 2.89%, respectively. Antimicrobial efficacy studies showed that levofloxacin-loaded PLGA microparticles inhibited bacterial growth. These results suggest that levofloxacin-loaded PLGA microparticles may be suitable for pulmonary administration.

KEYWORDS: Levofloxacin; drug delivery systems; w/o/o; surfactant type; PLGA; pulmonary delivery.

1. INTRODUCTION

Lower respiratory tract infections (LRIs) are a major cause of illness and mortality in both children and adults worldwide [1]. Eradication of LRIs with conventional antibiotics is difficult because a high dose is required for effective eradication of the organism. Therefore, there is an increasing interest in the direct administration of antibiotics into the respiratory tract by inhalation, as this achieves high concentrations in the lungs, improves antibacterial efficacy, and reduces toxicity due to low systemic exposure [2-4]. In recent years, some innovative formulations have emerged, including nanoparticles, microparticles, and liposomes, to improve compliance with inhalation therapy [5-9].

Aerodynamic size is a critical parameter for adequate deposition in the lungs. Aerosolized particles should have an aerodynamic size of 1-5 μm, which corresponds to the spherical equivalent geometric size of a particle with a density of 1 g/cm3[10-12].

Poly(lactide-co-glycolide) (PLGA) has been widely used to prepare sustained-release drug delivery systems for inhalation therapy [13-16]. The biodegradability and biocompatibility of PLGA are expected to minimize accumulation and effects on pulmonary function, respectively [17-19].

Levofloxacin has a broad spectrum of activity against Gram-positive and Gram-negative aerobic bacteria and atypical bacteria [20]. Levofloxacin is active against Streptococcus pneumoniae (including penicillin-resistant strains) and methicillin-susceptible Staphylococcus aureus. Gram-positive bacteria are more sensitive to levofloxacin than to ciprofloxacin. Levofloxacin is highly effective against Haemophilus influenzae, Moraxella catarrhalis and shows activity against Enterobacteriaceae [21]. Levofloxacin is widely used in clinical practice to treat various bacterial infections, including bone, joint, respiratory, urinary tract, and skin infections [22, 23]. Although levofloxacin is considered the safest of the fluoroquinolones due to its low rate of liver abnormalities, its common side effects involve the gastrointestinal tract and other organs [24]. Due to these side effects, encapsulation in drug delivery systems is potentially useful to achieve sustained

release of drugs and thus reduce their toxic concentrations. In this context, the use of PLGA-based drug delivery systems as vehicles for the safe administration of levofloxacin via inhalation therapy could be a viable therapeutic alternative.

Several encapsulation methods can be useful for various purposes, such as controlled delivery of drugs, masking the taste and odor of drugs, protecting drugs from degradation, and protecting the body from the toxic effects of drugs. One of the most common methods for preparing PLGA-based particles is the oil-in-water emulsion method [25-28]. Although this method can successfully incorporate water-insoluble drugs into the microparticles, it is not suitable for the encapsulation of highly water-soluble compounds. Successful encapsulation of such compounds requires high drug loading and predictable release from the microparticles. These problems can be overcome by using the double emulsion method. Various methods such as water in oil in water (w/o/w), solid in oil in water (s/o/w), water in oil in oil (w/o/o) and solid in oil in oil (s/o/o) have been investigated to produce stable formulations [29,30].

The aim of the present work is to develop and characterize PLGA-based microparticles loaded with levofloxacin for potential application in lung drug delivery. The effect of the type and concentration of surfactant in the inner or outer phase on the physicochemical properties and in vitro drug release profiles of the particles was investigated. The aerodynamic properties of the microparticles were also determined. Finally, the antibacterial capacity of the PLGA-based microparticles containing levofloxacin was evaluated.

2. RESULTS AND DISCUSSION

2.1. Determination of levofloxacin

Spectral scan – λmax of levofloxacin was found to be at 288 nm. The developed analytical method was validated according to the ICH guidelines [31]. Linearity is the capacity to obtain results directly proportional to the analyte concentration in the sample within a determined range. Linearity was investigated using levofloxacin solutions in both distilled water and pH 7.4 phosphate buffer solution containing 0.05% (w/v) Tween® 80 in seven different concentration levels ranging from 3 µg/ml to 9 µg/ml. The response of the drug was found to be linear in the investigation concentration range and the linear regression equations were y=72.264x+0.018 with correlation coefficient 0.998 for distilled water and y=72.202x+0.0016 with correlation coefficient 0.999 for pH 7.4 phosphate buffer solution containing 0.05% (w/v) Tween® 80.

The limit of detection (LOD) is defined as the minimum amount at which the analyte may be detected and is often determined by the analysis of samples with known analyte concentrations [31]. The LOD values were 0.122 µg/ml and 0.0156 µg/ml for distilled water and pH 7.4 phosphate buffer solution containing 0.05% (w/v) Tween® 80, respectively. The limit of quantification (LOQ) is a parameter of quantitative assays for low levels of compounds in the sample [31]. The LOQ values were 0.368 µg/ml and 0.0475 µg/ml for distilled water and pH 7.4 phosphate buffer solution containing 0.05% (w/v) Tween® 80, respectively.

Accuracy of an analytical procedure demonstrates a concordance degree between the value (accepted as conventionally true or an accepted reference value) and the obtained value [31]. Accuracy was determined by preparing levofloxacin solutions with known concentrations at three different levels. Recovery rate values for accuracy varied from 99.47% to 100.28% for distilled water and 100.35% to 101.38% for pH 7.4 phosphate buffer solution containing 0.05% (w/v) Tween® 80 meaning that good results were obtained.

The precision of an analytical procedure expresses the concordance between a series of measurements from multiple samplings of the same homogenous sample [31]. The intermediate precision (assays on different days) and repeatability (different assays on the same day) were determined with six scans of three levofloxacin solutions with known concentrations (3, 6 and 9 µg/ml) for both two media. Reproducibility values for intermediate precision (inter-day) and repeatability (intra-day) presented relative standard deviation lower than 1.5%.

2.1. Preparation of levofloxacin-loaded PLGA microparticles

Levofloxacin is a water-soluble drug. Thus, a modified w/o/o multiple emulsion solvent evaporation method was used to prepare levofloxacin loaded PLGA microparticles in this study for the first time. PLGA was chosen as a polymer because of its biocompatibility and biodegradability properties.

Different types and concentrations of surfactants were used in the inner aqueous or outer oily phase and the effect of these on the physicochemical characteristics and in vitro drug release profiles of particles were investigated. For this reason, four different types of Tween (Tween® 20, 40, 60 and 80) were used in the inner aqueous phase as surfactants to obtain a primary emulsion. Also, four different types of Span (Span® 20, 40, 60 and 80) were added to the outer oily phase as surfactants to obtain secondary emulsion (Table 1).

Table 1. Composition of levofloxacin-loaded PLGA microparticles.

Formulation code	Surfactant type in the inner phase	Surfactant concentration in the inner phase (% w/v)	Surfactant type in the outer phase	Surfactant concentration in the outer phase (% w/v)
F1	Tween® 20	0.5	Span® 80	1
F2	Tween® 20	1	Span® 80	1
F3	Tween® 20	3	Span® 80	1
F4	Tween® 40	0.5	Span® 80	1
F5	Tween® 40	1	Span® 80	1
F6	Tween® 40	3	Span® 80	1
F7	Tween® 60	0.5	Span® 80	1
F8	Tween® 60	1	Span® 80	1
F9	Tween® 60	3	Span® 80	1
F10	Tween® 80	0.5	Span® 80	1
F11	Tween® 80	1	Span® 80	1
F12	Tween® 80	3	Span® 80	1
F13	Tween® 60	3	Span® 20	0.5
F14	Tween® 60	3	Span® 20	1
F15	Tween® 60	3	Span® 20	3
F16	Tween® 60	3	Span® 40	0.5
F17	Tween® 60	3	Span® 40	1
F18	Tween® 60	3	Span® 40	3
F19	Tween® 60	3	Span® 60	0.5
F20	Tween® 60	3	Span® 60	1
F21	Tween® 60	3	Span® 60	3
F22	Tween® 60	3	Span® 80	0.5
F23	Tween® 60	3	Span® 80	3

2.2. Characterization of PLGA-based microparticles

2.2.1. Particle size and distribution

Surfactant type and concentration used in inner and outer phases influence particle size and distribution of microparticles [32]. In our study, we investigated the effect of types and concentrations of Tween as a surfactant in the inner aqueous phase on the particle size of levofloxacin loaded PLGA-based microparticles. Generally, the particle size of microparticles decreased as Tween concentration increased in the inner aqueous phase. In PLGA-based microparticles prepared with Tween® 20, increasing the concentration of surfactant in the aqueous inner phase from 0.5% (w/v) (F1) to 1% (w/v) (F2) did not cause a significant change ($p < 0.05$) in particle size. Besides, increasing the concentration of Tween® 20 to 3% (w/v) (F3) resulted in a significant reduction ($p < 0.05$) in particle size. Similar results were obtained for microparticles prepared with Tween® 40 and Tween® 60. In PLGA-based microparticles prepared with Tween® 80, increasing the surfactant concentration from 0.5% (w/v) (F10) to 1% (w/v) (F11) and 3% (w/v) (F12) also resulted in a significant decrease in particle size (Table 2).

Table 2. Characterization of levofloxacin-loaded PLGA microparticles (values are the mean ± standard deviations, n=3).

Formulation code	Particle size (µm)	Span value	% Encapsulation efficency	% Product yield
F1	29.57 ± 1.39	1.73 ± 0.22	58.47 ± 0.024	82.86
F2	29.40 ± 2.06	1.67 ± 0.04	70.71 ± 0.031	72.56
F3	7.03 ± 1.47	3.05 ± 0.43	75.42 ± 0.014	68.91
F4	36.26 ± 0.52	1.86 ± 0.18	71.41 ± 0.024	72.29
F5	33.65 ± 1.55	1.82 ± 0.14	73.36 ± 0.013	71.19
F6	10.40 ± 1.89	4.08 ± 0.49	80.67 ± 0.011	76.14
F7	13.0 ± 0.54	1.68 ± 0.05	89.47 ± 0.014	64.81
F8	10.69 ± 0.99	0.83 ± 0.13	87.62 ± 0.012	73.90
F9	5.59 ± 0.54	2.78 ± 0.23	82.51 ± 0.024	81.71
F10	50.47 ± 0.83	1.74 ± 0.20	57.81 ± 0.011	95.57
F11	29.21 ± 0.30	1.71 ± 0.28	63.44 ± 0.013	98.73
F12	10.59 ± 0.12	0.71 ± 0.04	68.61 ± 0.012	88.71
F13	7.01 ± 1.49	8.42 ± 2.78	45.26 ± 0.014	73.55
F14	9.31 ± 0.50	5.73 ± 1.24	37.54 ± 0.018	71.75
F15	58.10 ± 1.30	2.77 ± 0.59	54.46 ± 0.013	78.65
F16	76.19 ± 2.20	1.78 ± 0.04	27.90 ± 0.011	79.67
F17	9.57 ± 1.87	3.27 ± 0.06	67.87 ± 0.014	74.81
F18	45.58 ± 4.57	2.34 ± 0.10	64.59 ± 0.017	75.57
F19	52.63 ± 3.58	1.99 ± 0.16	29.72 ± 0.028	85.51
F20	20.84 ± 2.83	3.01 ± 0.34	66.43 ± 0.017	82.61
F21	29.18 ± 2.97	1.67 ± 0.15	78.43 ± 0.018	83.80
F22	15.99 ± 0.61	1.84 ± 0.34	52.78 ± 0.021	73.45
F23	11.86 ± 1.18	3.47 ± 0.29	82.89 ± 0.013	74.97

The effect of surfactant concentration on the droplet sizes and thus the mean size of microparticles can be explained by considering the interfacial tension. Interfacial tension decreases with increasing concentrations of surfactant, thus allowing for more efficient droplet break-up during emulsification [33,34]. Furthermore, the surfactant concentration has an important role in the emulsification process and in the protection stabilization of the droplets in the emulsification-evaporation method [30]. When emulsion droplets were formed, surfactant molecules are adsorbed between droplet surfaces and prevent aggregation and thus the mean size of the particles decreases. According to the results reported by previous studies, increasing surfactant concentration led to a decrease in particle size [35,36].

Also, the impact of surfactant type in the inner aqueous phase on the size of the PLGA-based microparticles was investigated. For this reason, different surfactant types (Tween® 20, 40, 60, and 80) were used in the inner aqueous phase to prepare microparticles. When the concentration of surfactant in the inner aqueous phase was kept constant at 3% (w/v), the smallest microparticles were formed in the F9 coded formulation prepared using Tween® 60 (d=5.59±0.54 µm) whereas the largest microparticles were formed in the F12 coded formulation prepared using Tween® 80 (d=10.59 ± 0.12 µm). As a result, there seemed to be no strong correlation between the particle size and the type of surfactant in this study. Similarly, previous studies indicated that the mean size of the PLGA microspheres was not affected by the type of surfactant used for the primary emulsion [34,37].

Four different Span types and three different concentrations for each Span type were tested to investigate the effect of Span type and concentration used as a surfactant in the outer oily phase on particle size. In these formulations, the surfactant type and concentration in the inner aqueous phase were kept constant as Tween® 60, 3% w/v. There is generally no correlation between the Span type and concentration used in the outer oily phase and the particle size of microparticles. The increase in surfactant concentration in the outer oily phase in formulations prepared with Span® 20 resulted in an increase in particle size (Table 2). However, there was no correlation between surfactant concentration and particle size in formulations prepared with Span® 40. When the Span® 40 concentration in the outer oily phase was increased from 0.5% (w/v) to 1% (w/v), the particle size of the PLGA-based microparticles decreased from 76.19 ± 2.20 µm (F16) to 9.57 ± 1.87 µm (F17). However, when the Span® 40 concentration was increased to 3% (w/v), the particle size was 45.58 ± 4.57 µm (F18). Similarly, results were obtained in particles prepared with Span® 60 and Span® 80 (Table 2). Increasing the concentration of Span® 60 or Span® 80 from 0.5% (w/v) to 1% (w/v) provided a decrease in particle size, while increasing the surfactant concentration to 3% (w/v) resulted in an increase in particle size.

Span values were calculated as an indicator of the particle size distribution of microparticles. In general, the smaller span values indicate that the particle size distribution of levofloxacin loaded PLGA-based microparticles is narrow (Table 2).

2.2.2. Encapsulation efficiency

High encapsulation efficiency is one of the characteristics of a successful drug delivery system to reduce the quantity of the carrier required for treatment [38]. In the present study, levofloxacin was encapsulated into PLGA-based microparticles by w/o/o double emulsification method for the first time. Because the outer phase was oil, levofloxacin did not diffuse into the outer phase and therefore high encapsulation efficiency values (up to 90%) were achieved. Gaspar et al. have prepared levofloxacin-loaded PLGA microspheres by using the w/o/w double emulsion-solvent evaporation method with a premix membrane homogenization step [39]. Because the outer phase is water in the w/o/w emulsification method, they have obtained lower encapsulation efficiency values (0.90-44.4%) compared with our results.

In formulations prepared with Tween® 20, the increased concentration of surfactant increased the encapsulation efficiency. The encapsulation efficiency in the formulation prepared with 0.5% w/v Tween® 20 (F1) is 58.47%, while the encapsulation efficiency in the formulation prepared with 3% w/v Tween® 20 (F3) is 75.42%. Similar results were obtained by Rojas et al. [40]. The amount of Tween® 20 affected both loading and encapsulation efficiency. The addition of Tween® 20 in the inner aqueous phase improved the encapsulation of β-lactoglobulin in PLGA microparticles. It is believed that this increase in encapsulation efficiency is due to the increase in stability of the primary emulsion. Several authors indicated that the stability of the primary emulsion is a pre-requisite for the successful loading of drugs in microparticles prepared by the double emulsion method [41, 42]. Increasing the stability of the primary emulsion reduced the diffusion of the active substance into the outer phase. The entrapment of the active substance in the inner phase resulted in a higher encapsulation efficiency. Similarly, in formulations prepared with Tween® 40 (F4, F5 and F6) and Tween® 80 (F10, F11 and F12), an increase in the concentration of surfactant in the inner aqueous phase resulted in an increase in encapsulation efficiency ($p<0.05$). In addition to this, in the formulations prepared with Tween® 60, it was observed that the encapsulation efficiency decreased slightly (Table 2).

In general, the increase in Span concentration in the outer phase resulted in an increase in encapsulation efficiency (Table 2). In formulations prepared with Span® 20, increasing concentration of the surfactant in the outer oily phase from 0.5% w/v to 1% w/v resulted in a slight decrease in the encapsulation efficiency, while an increase in the encapsulation efficiency was seen when increasing the concentration of the surfactant from 1% w/v to 3% w/v ($p<0.05$). In formulations prepared with Span® 40, increasing concentration of the surfactant in the outer oily phase from 0.5% w/v to 1% w/v resulted in a significant increase in the encapsulation efficiency, while a slight decrease in the encapsulation efficiency was seen when increasing concentration of the surfactant from 1% w/v to 3% w/v. However, this decrease was not statistically significant ($p>0.05$). In formulations prepared with Span® 60 (F19, F20 and F21) and Span® 80 (F8, F22 and F23), the encapsulation efficiency was increased in proportion to the concentration of surfactant in the outer oily phase (Table 2). This is the reason that as the concentration of surfactant in the outer oily phase increases, the stability of the seconder emulsion increases. Increased secondary emulsion stability prevented the diffusion of the active substance to the external phase and allowed it to be trapped in the inner phase. Dinarvand et al. also indicated that stabilizing secondary emulsion led to the highest drug loading [43].

2.2.3. Production yield

A production yield of range 64.81 – 98.73% (Table 2) in the PLGA-based formulations were obtained. Depending on the type of surfactant, an increase in the number of solid materials adhering to the container occurred in some formulations and this caused a decrease in production yield. The highest production yield value was obtained when Tween® 80 was used in the inner phase to prepare microparticles (F11).

2.2.4. In vitro drug release

Levofloxacin release profiles were investigated to evaluate the effect of the surfactant type and concentration in the inner or outer phase on the release of levofloxacin from PLGA-based microparticles. The release of levofloxacin from PLGA-based microparticles was studied for 24 hours. According to the in vitro drug release profiles, 4.2-36.1% of levofloxacin were released within the first hour (Figures 1 and 2). This burst release is thought to be due to the fact that the drug in the hydrophilic property is retained on the surface of the particles, but also due to rapid diffusion from the particle structure with porous channels [44]. As seen on

the DSC thermogram, encapsulation of levofloxacin into microparticles caused the conversion of crystalline levofloxacin to its amorphous form. The amorphous form probably dissolves faster than the crystalline form because of greater intermolecular forces in the crystal [45]. However, the encapsulation of levofloxacin in the PLGA matrix provided a sustained release after the initial burst release.

PLGA 50:50 (MW:24.000-38.000) was used as a polymer in this study. PLGA with a 50:50 lactide to glycolide ratio is more advantageous than other PLGAs due to its fastest degradation rate thus resulting in a faster drug release [46]. Gaspar et al. have observed partial degradation of PLGA microspheres after one week in the release medium [43]. Wischke and Schwendeman have indicated that a 4–6-week release can be achieved by a 50:50 PLGA with a low to medium molecular weight (e.g., Resomer® RG 502 or 503) [42].

The f_2 similarity factor was calculated and evaluated to compare the dissolution profiles of formulations. Acceptable f_2 values are between 50 and 100. Two dissolution profiles are declared similar if the f_2 value is over 50. When the change in the drug release profile was examined by the concentration of surfactant used in the inner phase, the increase in the amount of Tween® 20 causes a significant decrease in the release rate (Figure 1). The f_2 of 31.48 showed that the dissolution profiles of the formulation prepared with 0.5% w/v Tween® 20 and the formulation prepared with 3% w/v Tween® 20 are not similar. As mentioned above, the increase in the amount of Tween® 20 resulted in a decrease in the particle size of microparticles. Depending on the reduced particle size, the release rate may be expected to increase due to the increased surface area. On the contrary, the release rate decreased with an increasing amount of surfactant. This behavior is thought to be the result of improved primary emulsion stability and thus a better drug dispersion in the polymeric matrix and a lower amount of drug molecules near the surface of microparticles [48]. However, f_2 values greater than 50 showed that the concentration difference did not have a significant effect on the release profile for formulations prepared with Tween® 40, 60 and 80. When the effect of the Tween type on the drug release profile was examined, the highest release rate was generally obtained in particles prepared with Tween® 20 (Figure 1). The f_2 values calculated for F1-F4, F1-F7, and F1-F10 were found to be 27.99, 34.73 and 38.54, respectively. These results showed that dissolution profiles of formulations prepared by different Tween types are significantly different. This may be since Tween® 20 has the highest HLB value (16.7), thus enhancing the environment hydrophilicity and facilitating the diffusion of the active compound.
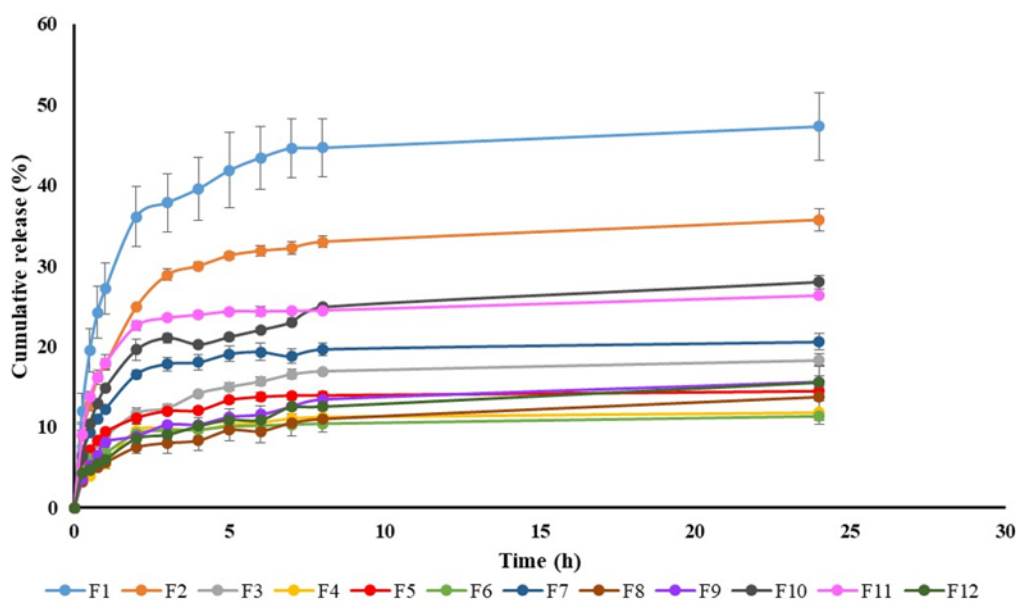

Figure.1. Effect of surfactant type and concentration in the inner aqueous phase on release profiles of levofloxacin from PLGA-based particles (mean ± SD, n=3).

When the effect of the outer phase surfactant on the drug release profile is examined, there is generally no correlation between the Span type or concentration and the drug release rate (Figure 2). In a study by Bolourtchian et al. [49], the use of Span at different concentrations in formulations is generally not effective at the rate of drug release. The reason for this is that Span's water solubility and wettability are low.
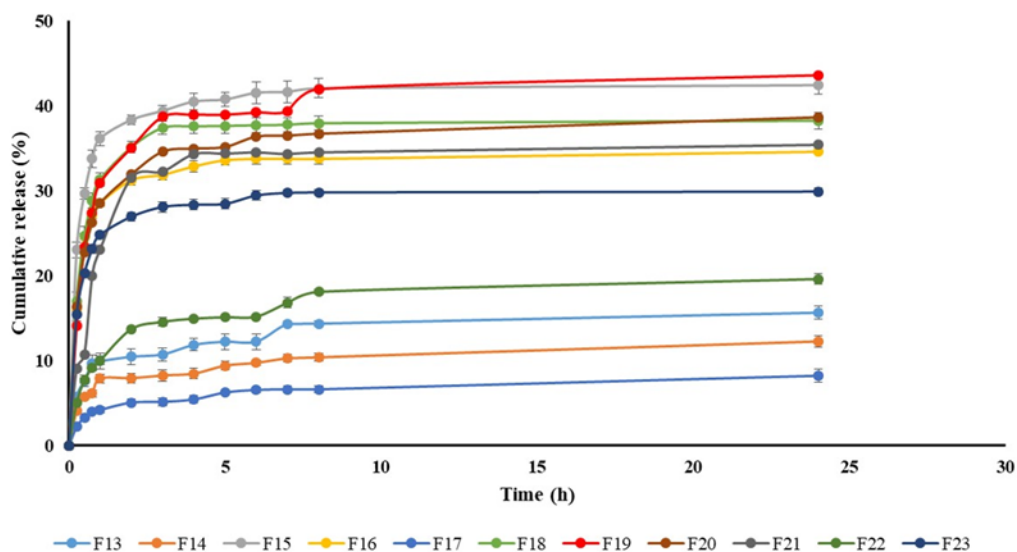
Figure 2. Effect of surfactant type and concentration in the outer oily phase on release profiles of levofloxacin from PLGA-based particles (mean ± SD, n=3).

The kinetic evaluation of the dissolution rate data was carried out according to zero-degree, first-degree, Higuchi, Rosin-Rammler-Sperling-Bennet-Weibull (RRSBW), and Hixson-Crowell kinetics, and the results of the evaluations are given in Table 3 and Table 4. Considering the data obtained for formulations, the highest r^2 values were obtained with RRSBW (Table 3 and Table 4). They were observed that the root mean square (RMS) value for RRSBW was also quite low. The fact that the β values calculated in Weibull kinetics were less than 1 indicated that they reached the plateau after the initial rapid release of the active substance, similar to the first-order kinetics, and parabolic dissolution rate profiles were obtained [50].

Table 3. In vitro release kinetics of levofloxacin-loaded PLGA microparticles prepared by different surfactant types and concentrations in the inner aqueous phase.

		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Zero order	r^2	0.389	0.421	0.481	0.398	0.355	0.357	0.352	0.630	0.559	0.478	0.307	0.618
	k_0	1.425	1.129	0.593	0.369	0.397	0.298	0.583	0.457	0.504	0.863	0.674	0.522
	SD	0.538	0.400	0.186	0.138	0.161	0.121	0.238	0.105	0.135	0.272	0.305	0.124
	RMS	142.018	78.231	16.888	9.288	12.737	7.142	27.839	5.453	8.939	36.232	45.613	7.499
First order	r^2	0.443	0.457	0501	0.404	0.368	0.368	0.369	0.651	0.581	0.517	0.329	0.640
	k_1	0.021	0.015	0007	0.004	0.004	0.003	0.007	0.005	0.006	0.011	0.008	0.006
	SD	0.007	0.005	0002	0.001	0.002	0.001	0.003	0.001	0.001	0.003	0.004	0.001
	RMS	0.025	0.012	0002	0.001	0.001	0.001	0.003	0.001	0.001	0.005	0.006	0.001
Higuchi	r^2	0.719	0.750	0804	0.721	0.682	0.674	0.680	0.902	0.852	0.786	0.621	0.896
	k_h	9.630	7.498	3.810	0.248	2.734	2.038	4026	2.718	3.095	5.501	4.764	3.126
	SD	1.816	1.305	0.568	0.465	0.563	0.427	0.833	0.269	0.389	0.867	1.122	0.320
	RMS	65.373	33.757	6.385	4.285	6.277	3.620	13.764	1.439	2.999	14.887	24.943	2.033
Weibull	r^2	0.892	0.916	0.951	0.884	0.904	0.913	0.878	0.985	0.947	0.901	0.832	0.981
	β	0.355	0.363	0.330	0.326	0.240	0.214	0.280	0.327	0.326	0.333	0.243	0.329
	SD	0.039	0.035	0.024	0.037	0.025	0.021	0.033	0.013	0.024	0.035	0.035	0.015
	τ_d	0.945	0.957	0.975	0.940	0.951	0.955	0.937	0.992	0.973	0.949	0.912	0.990
	RMS	0.006	0.004	0.002	0.005	0.002	0.002	0.004	0.001	0.002	0.004	0.004	0.001
Hixon-Crowell	r^2	0.194	0.214	0.230	0.207	0.165	0.159	0.175	0.274	0.248	0.220	0.150	0.275
	k_h	2.882	0.064	0.052	0.044	0.041	0.037	0.048	0.049	0.050	0.058	0.048	0.052
	SD	1.769	0.037	0.029	0.026	0.028	0.026	0.032	0.024	0.026	0.033	0.035	0.025
	RMS	35.881	0.675	0.407	0.330	0.387	0.324	0.492	0.289	0.339	0.540	0.595	0.316

Table 4. In vitro release kinetics of levofloxacin-loaded PLGA microparticles prepared by different surfactant types and concentrations in the outer oily phase.

		F13	F14	F15	F16	F17	F18	F19	F20	F21	F22	F23
Zero order	r^2	0.438	0.484	0.207	0.215	0.541	0.217	0.319	0.290	0.300	0.473	0.219
	k_0	0.429	0.346	0.843	0.714	0.252	0.826	1.122	0.919	1.035	0.604	0.620
	SD	0.146	0.108	0.498	0.411	0.070	0.473	0.494	0.433	0.477	0.192	0.353
	RMS	10.484	5.695	121.365	82.821	2.391	109.565	119.635	91.922	111.392	18.116	61.090
First order	r^2	0.460	0.502	0.243	0.242	0.553	0.242	0.368	0.332	0.319	0.497	0.242
	k_1	0.005	0.004	0.012	0.009	0.003	0.011	0.016	0.013	0.014	0.007	0.008
	SD	0.002	0.001	0.006	0.005	0.001	0.006	0.006	0.005	0.006	0.002	0.004
	RMS	0.001	0.001	0.020	0.012	0.000	0.018	0.020	0.014	0.017	0.002	0.009
Higuchi	r^2	0.734	0.774	0.482	0.495	0.833	0.509	0.630	0.597	0.620	0.786	0.504
	k_h	2.757	2.177	6.397	5.387	1.552	6.284	7.840	6.549	7.399	3.871	4.678
	SD	0.501	0.355	2.000	1.640	0.209	1.863	1.810	1.624	1.747	0.610	1.399
	RMS	4.972	2.492	79.285	53.270	0.868	68.773	64.921	52.257	60.518	7.363	38.801
Weibull	r^2	0.917	0.931	0.812	0.753	0.950	0.764	0.829	0.864	0.787	0.924	0.814
	β	0.214	0.229	0.153	0.166	0.274	0.193	0.264	0.210	0.363	0.309	0.156
	SD	0.020	0.020	0.023	0.030	0.020	0.034	0.038	0.026	0.060	0.028	0.024
	τ_d	0.958	0.965	0.901	0.868	0.975	0.874	0.910	0.930	0.887	0.961	0.902
	RMS	0.002	0.001	0.002	0.003	0.001	0.004	0.005	0.003	0.013	0.003	0.002
Hixon-Crowell	r^2	0.177	0.193	0.102	0.108	0.225	0.113	0.152	0.134	0.176	0.216	0.108
	k_h	0.043	0.041	0.047	0.045	0.038	0.048	0.058	0.052	0.061	0.051	0.043
	SD	0.028	0.025	0.042	0.039	0.021	0.041	0.041	0.040	0.040	0.030	0.037
	RMS	0.375	0.306	0.872	0.759	0.224	0.819	0.826	0.773	0.765	0.427	0.688

2.3. The selected PLGA-based microparticle formulation (F9) was subjected to further investigation

The particle size, encapsulation efficiency and production efficiency values were taken into consideration in the selection of PLGA-based microparticle formulation to be used in the following studies. Thus, F9 coded formulation with 5.59 ± 0.54 μm in particle size, which is in an acceptable range for pulmonary delivery, 82.51 ± 0.024% in EE% and 81.71% in production yield were chosen for the following studies.

2.4. Characterization of selected PLGA-based microparticle formulation

2.4.1. Morphology of PLGA-based microparticles

The external surface morphology of the microparticles was analyzed by FE-SEM. As seen in Figure 3, microparticles have a spherical shape with uniform surface morphology. No pores were observed on the smooth surface of microparticles.

Figure 3. SEM image of levofloxacin loaded PLGA particles (F9).

2.4.2. Zeta potential of PLGA-based microparticles

The zeta potential is critical for the stability of particles in the dispersed state. When the zeta potential values of particles are close to ±30 mV, the colloidal systems form stable dispersions with no aggregate formation observed [51]. The zeta potential values of levofloxacin-loaded PLGA microparticles were -24.93±0.68 mV. This result showed that the particles had good colloidal stability and did not exhibit aggregation tendency.

2.4.3. Thermal analysis

Differential scanning calorimetry (DSC) is a thermal analysis method that measures the change in physical properties of samples, along with temperature against time [52]. In this study, thermal analysis using DSC was performed to determine the change in the physical state of levofloxacin after encapsulation. DSC thermograms of the levofloxacin, PLGA and levofloxacin-loaded microparticles (F9) is given in Figure 4. The pure endothermic peak (45.7 °C) in the pure PLGA shows that PLGA has an amorphous structure. Figure 4 illustrates that levofloxacin gives a sharp endothermic peak at 235.87 °C. This thermal event could be ascribed to the melting of the crystal form of levofloxacin. Similar results were obtained in previous studies [53,54]. However, the absence of an endothermic peak related to levofloxacin in the microparticles indicates that active substances turned amorph form and be trapped in microparticles [55,56].
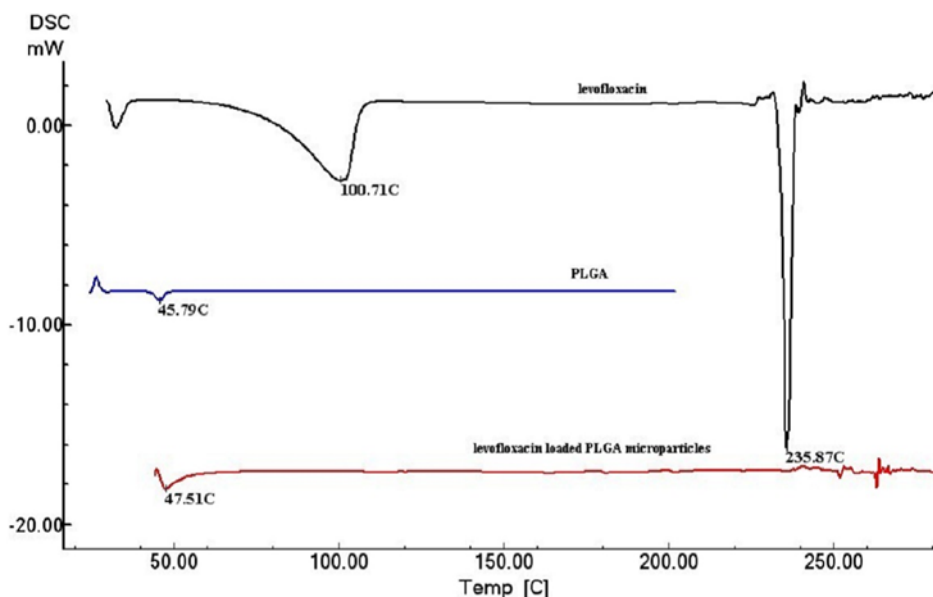
Figure 4. DSC thermograms of levofloxacin; PLGA and F9.

2.4.4. Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectral data are used to confirm the chemical stability of levofloxacin after the preparation of PLGA-based microparticles. FTIR spectra for pure levofloxacin, PLGA and levofloxacin-loaded microparticles (F9) was shown in Figure 5. The FTIR of levofloxacin showed the following characteristic peaks at 3242 cm^{-1} due to carboxylic group, in between 2801-2933 cm^{-1} due to alkanes group stretching, 1718cm^{-1} due to stretching of carbonyl group, 1045 cm^{-1} due to the presence of halogen group. This result was found to be compatible with previous studies [57]. Although there was a small decrease in the intensity of the peaks in the FTIR spectra of levofloxacin-loaded microparticles due to a partial loss of drug crystallinity in microparticles, the peaks remained almost the same. These results showed that the drug is physically dispersed in the polymeric matrix.

Changes generated after damaging exposition onto different factors (e.g., light, humidity, temperature, and pH) cause transformations with functional groups that show characteristic fingerprints in the IR spectrum [58]. Therefore, FTIR is a valuable tool for monitoring the stability of drugs in the solid state. In this study, FTIR spectroscopy has been used to investigate the stability of levofloxacin encapsulated into the PLGA-based microparticles. No significant changes between the spectra of fresh microparticles and microparticles stored at 25°C ± 2°C/40% RH ± 5% over 1 year were observed. This result demonstrates that levofloxacin encapsulated into PLGA-based microparticles remained stable over the time period of 1 year.
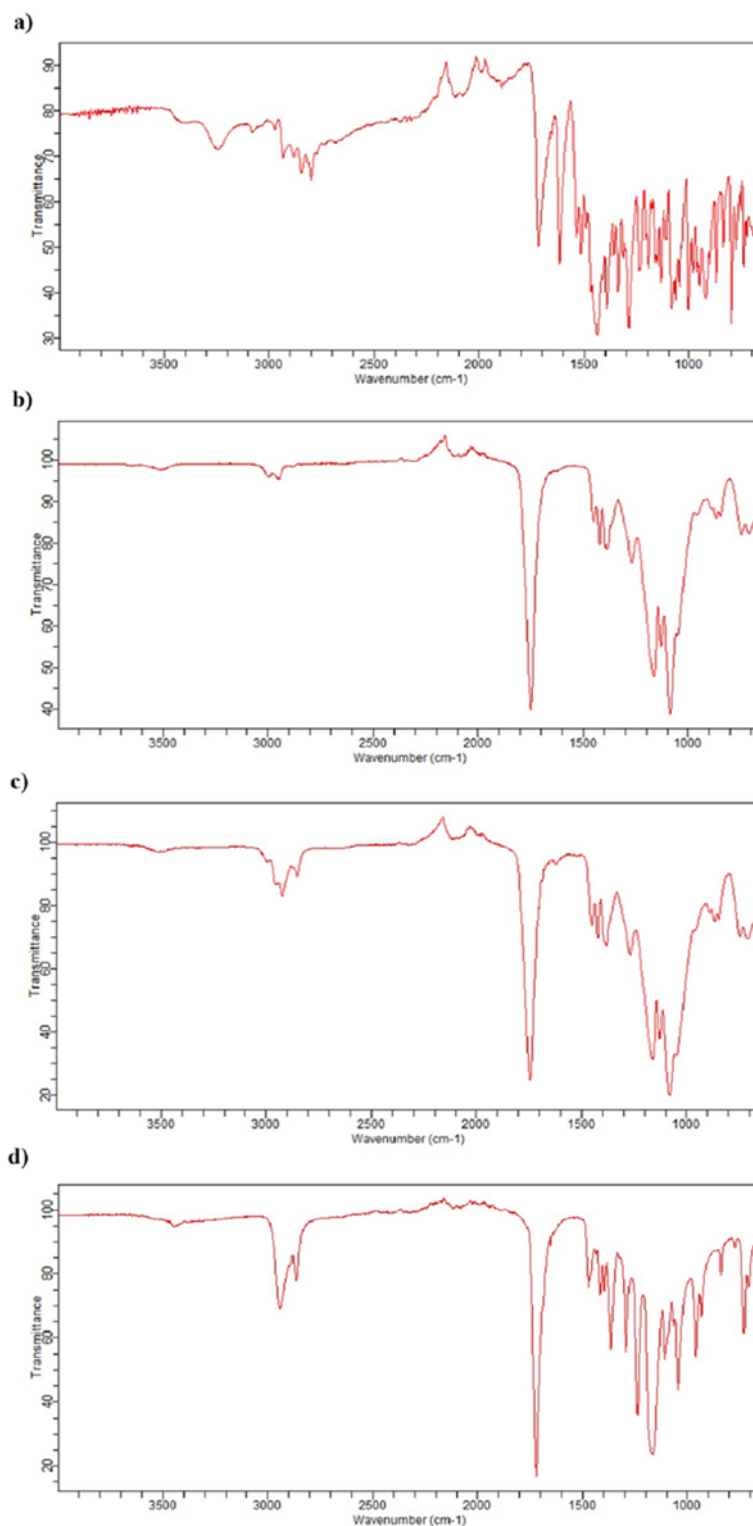
Figure 5. FTIR spectra of a) levofloxacin; b) PLGA; c) fresh F9 and d) stored F9.

2.4.5. Aerodynamic characteristics

The pulmonary route is one of the most preferred routes for drug delivery because it provides a high surface area for quicker and effective drug absorption through the pulmonary tract. Current inhalable therapies have various limitations, including a short half-life because of pulmonary clearance, enzymatic degradation, and fast systemic absorption, followed by poor bioavailability of drugs at the target site, resulting in increased dosing frequency, insufficient therapeutic efficacy, and adverse effects. As a result, there is an urgent need for an efficacious inhalation therapy that could overcome these limitations to produce a sustained therapeutic effect. Three clinically proven and industrially viable pulmonary inhalational pharmaceutical dosage forms have been developed based on various classes of device: nebulizers, dry powder inhalers (DPIs), and pressurized metered-dose inhalers (pMDIs) (59). In this study, the inhaler system of microparticles was prepared as a DPI system considering their advantages over other inhalation systems. Aerolizer® inhaler was used as the device for the DPI system since it provides the opportunity of the relatively easy and effective administration of different doses [12].

There are several factors that affect the settling of inhalable particles in the airways, thereby influencing drug bioavailability. For formulations of inhalable particles, it is necessary to consider the aerodynamic properties of the particle, including its mass median aerodynamic diameter (MMAD), which has a major role in the accumulation of particles in the different regions of the airway [6, 12]. Generally, the particles that MMAD's higher than 10 µm are deposited in the oropharynx, between 5 and 10 µm in the central airways and from 1 to 5 µm in the small airways and alveoli. Therefore, aerosols for inhalation should be made of particles with aerodynamic diameters between 1 and 5 µm to ensure effective distribution in the deep lung [60]. According to the calculations, MMADa value of optimum microparticle formulation was found 5.44 ± 0.19 µm (Table 5). Furthermore, microparticles showed FPF of $50.99 \pm 2.89\%$ (Table 5) indicating that a consistent amount of formulation has an aerodynamic size ≤ 5 µm, and thus displays the appropriate size to reach the respiratory zone. Our results show that levofloxacin-loaded PLGA microparticles can be effectively deposited in the deep-lung (Figure 6). In summary, levofloxacin loaded PLGA microparticles are suitable for pulmonary delivery.

Table 5. Actual mass median aerodynamic diameter (MMADa) and fine particle fraction (FPF) (%) of PLGA-based particles after aerosolization (values are the mean ± standard deviations, n = 3).

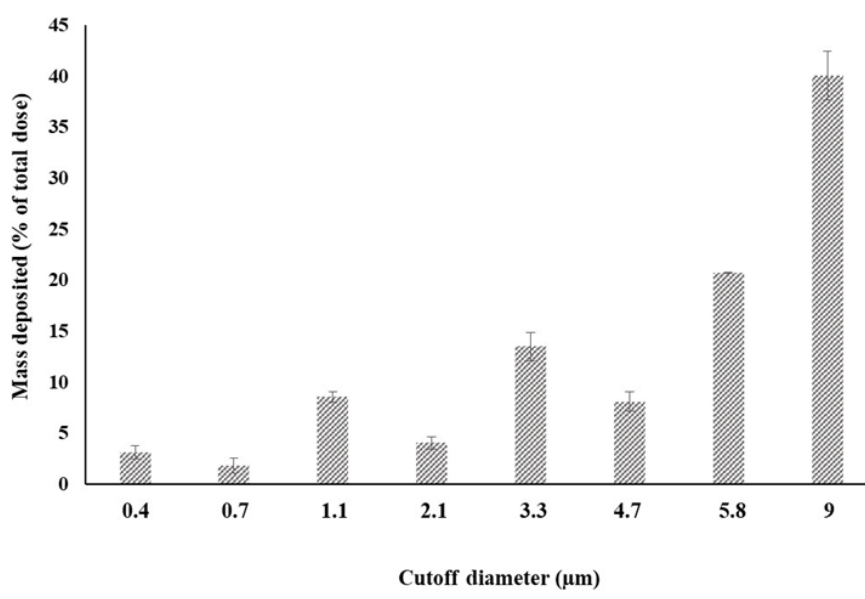| Formulation code	MMADa (µm)	FPF (%)
F9	5.44 ± 0.19 µm	50.99 ± 2.89

Figure 6. Deposition profile of levofloxacin-loaded PLGA particles following aerosolization into the Andersen cascade impactor.

2.4.6. Antimicrobial efficacy test

The antimicrobial efficacy study was performed with F9 coded formulation. The antimicrobial efficacy of levofloxacin released from PLGA-based microparticles at different time points was compared using the same amount of free levofloxacin as a reference. As shown in Figure 7 and Table 6, there are no differences between the size of inhibition zones of levofloxacin released from microparticles and that of free levofloxacin. Furthermore, inhibition zone sizes increased due to increased levofloxacin content in samples taken from dissolution medium at different time points (Table 6). According to the statistical analysis results, the zone sizes of levofloxacin released from microparticles at 8 h and 24 h were found to be significantly higher ($p < 0.05$) than that of 1 h for Klebsiella pneumonia. Furthermore, significant increases ($p < 0.05$) were obtained in the zone sizes due to increasing time at all time intervals for Escherichia coli.

Because of the sustained release of the drug from microparticles, the amount of released drug is going to be lower when compared with free drug. However, the PLGA-based microparticles could be helpful in achieving a similar therapeutic effect at lower doses providing evidence of enhanced drug deposition and increased therapeutic index by sustaining the therapeutic action in the lung.

Table 6. Inhibition zones and efficiencies (%) of F9 coded formulations for some bacterias that causes infection in lungs according to the results of antimicrobial efficacy test (n=5).

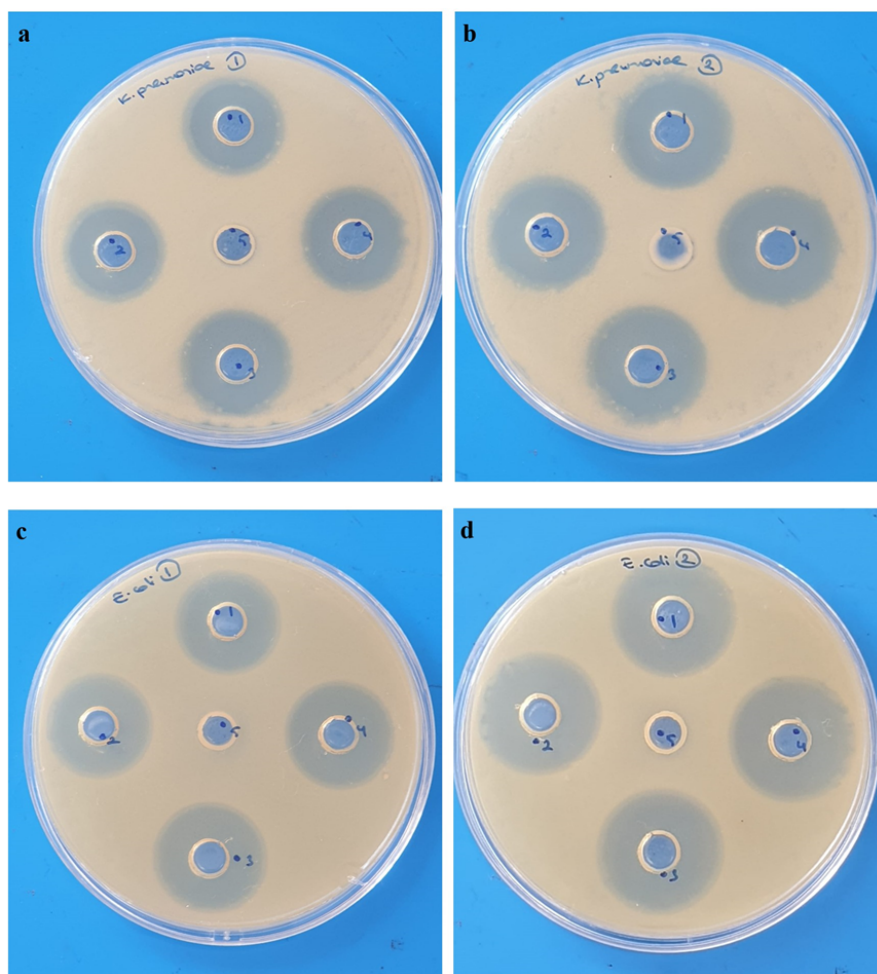|	*Klebsiella pneumonia*		*Escherichia coli*	
	Zone diameter (mm±SD)		Zone diameter (mm±SD)	
Time (h)	Free levofloxacin	F9	Free levofloxacin	F9
1	19.00±0.00	19.00±0.00	20.20±0.45	20.20±0.45
4	20.00±0.00	20.00±0.00	22.40±0.55	22.40±0.55
8	21.60±1.34	21.60±1.34	24.60±0.55	24.60±0.55
24	21.20±0.45	21.20±0.45	25.80±0.45	25.80±0.45

Figure 7. Antimicrobial efficacy of free levofloxacin and levofloxacin released from PLGA-based particles at different time points against Klebsiella pneumoniae (a and b) and Escherichia coli (c and d) (n=5).
1: levofloxacin released from PLGA-based particles at 1 h; 2: free levofloxacin equal to the amount of levofloxacin released at 1 h; 3: levofloxacin released from PLGA-based particles at 4 h; 4: free levofloxacin equal to the amount of levofloxacin released at 4 hours in a and c.
1: levofloxacin released from PLGA-based particles at 8 h; 2: free levofloxacin equal to the amount of levofloxacin released at 8 h; 3: levofloxacin released from PLGA-based particles at 24 h; 4: free levofloxacin equal to the amount of levofloxacin released at 24 hours in b and d.
Sterilized PBS was applied to the 5 coded wells as a negative control.

3. CONCLUSION

The PLGA-based microparticles containing levofloxacin were prepared successfully by using the w/o/o double emulsion solvent evaporation method. By varying the type and concentration of surfactant in the inner aqueous phase or outer oily phase, particles with suitable particle size and encapsulation efficiency could be obtained. According to the results of in vitro characterization studies, F9 coded formulation was thought as optimal formulation. Their aerodynamic properties showed that levofloxacin-loaded PLGA microparticles can be effectively deposited in the deep lung. Antibacterial activity test results have shown that microparticles were found to be active against pathogens causing lung infections. As a result, PLGA-based microparticles containing levofloxacin can be suitable for pulmonary delivery.

4. MATERIALS AND METHODS

4.1. Materials

The levofloxacin was kindly obtained from Koçak Farma (Turkey). Poly (lactide-co-glycolide (PLGA 50:50, MW: 24000–38000), poly (vinyl alcohol) (PVA), dichloromethane (99.9%, HPLC grade), n-hexan, ethyl acetate (99.5%, HPLC grade) and acetonitrile were from Sigma (Germany). All the other chemicals used were of analytical grade.

4.2. Methods

4.2.1. Determination of levofloxacin

To detect and quantification of levofloxacin, a spectrophotometric method was developed. An ultraviolet-visible/visible spectrophotometer (Thermo Scientific, Germany) with matched 1 cm quartz cells (cuvettes) was used for recording the absorption spectra. Stock solutions of levofloxacin containing 1 mg/ml were prepared in distilled water or pH 7.4 phosphate buffer solution containing 0.05% (w/v) Tween® 80. Standard dilutions were prepared to vary between 3-9 µg/ml. The developed method was validated based on the International Council for Harmonization guidelines Q2 (R1) [31]. Method validation was performed, including linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy and precision.

4.2.2. Preparation of PLGA-based microparticles

Levofloxacin-loaded PLGA-based microparticles were prepared by modified w/o/o emulsion-solvent evaporation technique [29,30,61,62]. As an inner phase, different Tween types (Tween® 20, Tween® 40, Tween® 60, and Tween® 80) were dissolved in distilled water in different concentrations (0.5, 1 and 3% w/v). 10 mg levofloxacin was dissolved in 1 ml of this solution. Polymer (PLGA) was dissolved in 5 ml of acetonitrile as an organic solvent. To prepare primary emulsion (w/o), the aqueous solution of levofloxacin and polymer solution in organic solvent homogenized in a high-speed homogenizer (Ultra Turrax T-25, Ika, Staufen, Germany) in 8000 rpm for 2 min. This primary emulsion poured into liquid paraffin containing different types and concentrations of Span, then homogenized at 8000 rpm for 5 min to obtain w/o/o secondary emulsion. This secondary emulsion is mixed in a magnetic stirrer at 450 rpm for 24 hours to evaporate organic solvent. Prepared particles were centrifuged at 9000 rpm for 20 minutes and washed with n-hexane and distilled water. These separated microparticles were dispersed in distilled water, frozen at −40 °C overnight and placed in the lyophilize equipment (Christ Gamma 2 -16 LSC, Quebec, CA) operating at 0.05 mmHg for 48 h. Dried particles were kept in sealed jars in a desiccator. The composition of levofloxacin-loaded PLGA microparticles were given in Table 1.

4.2.3. Characterization of PLGA-based microparticles

Particle size analysis

The particle size of PLGA-based microparticles was determined with a laser diffraction particle size analyzer (Sympatec Helos Model H0849, Clausthal-Zellerfeld, Germany). About 5 mg of dry particles were suspended in distilled water. The particle size is measured at 25±2 °C in triplicate. The mean particle size and particle size distribution were measured. The span value was calculated using Equation (1):

$$Span=(D90\%-D10\%)/(D50\%) \tag{1}$$

where DN% (N= 10, 50, 90) is the volume percentage of particles with diameters up to DN%, is equal to N%. The smaller span value indicates the narrower size distribution.

Encapsulation efficiency

The encapsulation efficiency of the lyophilized microparticles was determined by using the direct method. For this reason, 5 mg of dried PLGA-based particles were dissolved in acetonitrile and then ultrapure water (MilliQ Water, Merck Millipore, Darmstadt, Germany) was added. The mixture was shaken in order to

extract levofloxacin into aqueous phase from the organic phase. The encapsulation efficiency (%) was calculated using Equation (2).

Encapsulation efficiency (%) = (Calculated drug concentration)/ (Theoretical drug concentration) × 100 (2)

Production yield

PLGA-based microparticles were weighed at the end of preparation and the production yield (%) was calculated using Equation (3).

Production Yield (%) = (Total microparticle amount)/ (Total solid material amount) ×100 (3)

In vitro release studies

The in vitro release studies of levofloxacin from the PLGA-based microparticles were performed using the static method. 25 mg of levofloxacin-loaded microparticles were dispersed in 1 ml of pH 7.4 phosphate buffer solution containing 0.05% (w/v) Tween® 80 as a dissolution medium. The microparticles placed into the dialysis membrane (Sigma, 12.000 Da MWCO) were placed into the 100 ml dissolution medium and shaken horizontally in a shaking water bath (GFL 1083, Germany) at 100 rpm and 37±0.5°C. 3 ml samples were taken and 3 ml fresh release medium was added at specific time points. The taken samples were determined spectrophotometrically at a wavelength of 288 nm. All experiments were performed in triplicate. The percentage of levofloxacin released from the PLGA-based microparticles was defined as the mass of levofloxacin released divided by the total mass of levofloxacin in the microparticles.

4.2.4. The selected PLGA-based microparticle formulation (F9) was subjected to further investigation

Morphology of PLGA-based microparticles

To determine the surface morphologies of levofloxacin-loaded PLGA microparticles a scanning electron microscope was used (FE-SEM) (FEI Quanta Model 200F, Tokyo, Japan). Microparticles were mounted onto metal stubs to prepare samples. The PLGA-based microparticles were examined at 10–30 kV accelerating voltage after being vacuum-coated with a thin layer (100–150 A) of gold. The photomicrographs were then taken at a magnification of 3000–5000.

Zeta potential of PLGA-based microparticles

To determine the zeta potential of PLGA-based microparticles, zeta potential measurements were performed via electrophoretic light scattering for particles. All measurements were performed at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) in triplicate.

Thermal analysis

Thermal analysis of levofloxacin, PLGA and levofloxacin-loaded PLGA microparticles (F9) were performed by DSC (Shimadzu DSC-60, Kyoto, Japan). About 5 mg of each sample were crimped into an aluminium pan. Analysis was performed at a scanning temperature ranging from 25°C to 400°C at a heating rate of 5°C/min. The data obtained were processed on TA 60 universal analyzer software.

FTIR spectroscopy

Fourier transform infrared (FTIR) spectrums of levofloxacin, PLGA and levofloxacin-loaded PLGA microparticles (F9) were obtained by Shimadzu IRAffinity-1S spectrometer (Shimadzu, Kyoto, Japan). The spectra were recorded in the IR range from 400 to 4000 cm^{-1}. Furthermore, FTIR spectroscopy was employed to monitor the stability of levofloxacin encapsulated into the PLGA-based microparticles. For this purpose, FTIR spectra of fresh microparticles and microparticles stored at 25°C ± 2°C/40% RH ± 5% over 1 year were compared.

Capsule filling

PLGA-based microparticles was filled into hydroxypropylmethylcellulose (HPMC) capsules (size 3) with the weight of 20.0 ± 1.0 mg.

Aerodynamic characteristics

The aerodynamic characteristics of the levofloxacin-loaded PLGA microparticles were performed by eight-stage Mark II ACI (Copley Scientific Ltd., Nottingham, UK). A capsule containing microparticles was placed into the Aerolizer® DPI device and then the device was attached to the ACI. The capsule was punctured and the vacuum pump was switched on at 28 liters/min. [63-65].

Antimicrobial efficacy

The antimicrobial efficacy of the selected formulation (F9) was determined by the conventional agar diffusion method employing the cup plate technique against Klebsiella pneumoniae (RSKK 574) and Escherichia coli (ATCC 25922) [6, 66]. Antimicrobial efficacy assays were performed with samples released from the PLGA-based particles at different time points (1, 4, 8 and 24 hours). To compare the antimicrobial activity of drugs released from the particles with free drug, free levofloxacin equal to the amount of levofloxacin released at 1, 4, 8 and 24 hours were applied. The zone of inhibition (ZOI) measured for samples was compared with the sterilized PBS as a negative control. Each sample was tested five times.

4.2.5. Statistical analysis

Results were expressed as mean ± standard deviation (SD) from at least three separate measurements. The statistical difference was measured by using the one-way analysis of variance (ANOVA) followed by the post hoc Tukey multiple comparison test. All analyses were performed by SPSS for Windows statistical software version 11.0. Significance was established when the P-value was <0.05.

REFERENCES

[1] Traini D, Young PM. Delivery of antibiotics to the respiratory tract: an update. Expert Opin Drug Deliv. 2009;6(9):897–905. [CrossRef]

[2] Merchant Z, Taylor KMG, Stapleton P, Razak SA, Kunda N, Alfagih I, Sheikh K, Saleem IY, Somavarapu S. Engineering hydrophobically modified chitosan for enhancing the dispersion of respirable microparticles of levofloxacin. Eur J Pharm Biopharm. 2014;88(3):816–829. [CrossRef]

[3] Learoyd TP, Burrows JL, French E, Seville PC. Chitosan-based spray-dried respirable powders for sustained delivery of terbutaline sulfate. Eur J Pharm Biopharm. 2008;68(2):224–234. [CrossRef]

[4] Ungaro F, d'Angelo I, Coletta C, d'Emmanuele di Villa Bianca R, Sorrentino R, Perfetto B, Tufano MA, Miro A, La Rotonda MI, Quaglia F. Dry powders based on PLGA nanoparticles for pulmonary delivery of antibiotics: Modulation of encapsulation efficiency, release rate and lung deposition pattern by hydrophilic polymers. J Control Release. 2012;157(1):149–159. [CrossRef]

[5] Desu HR, Thoma LA, Wood GC. Nebulization of cyclic arginine-glycine-(d)-aspartic acid-peptide grafted and drug encapsulated liposomes for inhibition of acute lung injury. Pharm Res. 2018;35(5):94. [CrossRef]

[6] Topal GR, Devrim B, Eryilmaz M, Bozkir A. Design of ciprofloxacin-loaded nano-and microcomposite particles for dry powder inhaler formulations: preparation, in vitro characterisation, and antimicrobial efficacy. J Microencapsul. 2018;35(6):533–547. [CrossRef]

[7] Ubale R V, Shastri PN, Oettinger C, D'Souza MJ. Pulmonary administration of microparticulate antisense oligonucleotide (aso) for the treatment of lung inflammation. AAPS PharmSciTech. 2018;19(4):1908–1919. [CrossRef]

[8] Pulivendala G, Bale S, Godugu C. Inhalation of sustained release microparticles for the targeted treatment of respiratory diseases. Drug Deliv Transl Res. 2020;10:339-353. [CrossRef]

[9] Puri V, Chaudhary KR, Singh A, Singh C. Inhalation potential of N-Acetylcysteine loaded PLGA nanoparticles for the management of tuberculosis: In vitro lung deposition and efficacy studies. Curr Res Pharmacol Drug Discov. 2022;3:100084. [CrossRef]

[10] Ungaro F, d'Emmanuele di Villa Bianca R, Giovino C, Miro A, Sorrentino R, Quaglia F, La Rotonda MI. Insulin-loaded PLGA/cyclodextrin large porous particles with improved aerosolization properties: In vivo deposition and hypoglycaemic activity after delivery to rat lungs. J Control Release. 2009;135(1):25–34. [CrossRef]

[11] Telko MJ, Hickey AJ. Dry Powder Inhaler Formulation. Respir Care. 2005;50(9):1209 – 1227. [CrossRef]

[12] Devrim B, Bozkır A, Canefe K. Preparation and evaluation of poly(lactic-co-glycolic acid) microparticles as a carrier for pulmonary delivery of recombinant human interleukin-2: II. In vitro studies on aerodynamic properties of dry powder inhaler formulations. Drug Dev Ind Pharm. 2011;37(11):1376–1386. [CrossRef]

[13] Doan TVP, Couet W, Olivier JC. Formulation and in vitro characterization of inhalable rifampicin-loaded PLGA microspheres for sustained lung delivery. Int J Pharm. 2011;414(1):112–117. [CrossRef]

[14] Feng T, Tian H, Xu C, Lin L, Xie Z, Lam MH-W, Liang H, Chen X. Synergistic co-delivery of doxorubicin and paclitaxel by porous PLGA microspheres for pulmonary inhalation treatment. Eur J Pharm Biopharm. 2014;88(3):1086–1093. [CrossRef]

[15] Ramazani F, Chen W, Van Nostrum CF, Storm G, Kiessling F, Lammers T, Hennink WE, Kok RJ. Formulation and characterization of microspheres loaded with imatinib for sustained delivery. Int J Pharm. 2015;482(1):123–130. [CrossRef]

[16] Gaspar MC, Grégoire N, Sousa JJS, Pais AACC, Lamarche I, Gobin P, Olivier JC, Marchand S, Couet W. Pulmonary pharmacokinetics of levofloxacin in rats after aerosolization of immediate-release chitosan or sustained-release PLGA microspheres. Eur J Pharm Sci. 2016;93:184–191. [CrossRef]

[17] Devrim B, Alemdar M. The application of different microencapsulation methods and formulation parameters on antibiotic loaded PLGA microparticles for pulmonary delivery. Turkish J Pharm Sci. 2016;13(1):119–133. [CrossRef]

[18] Kalelkar PP, Moustafa DA, Riddick M, Goldberg JB, McCarty NA, García AJ. Bacteriophage-loaded poly(lactic-co-glycolic acid) microparticles mitigate staphylococcus aureus infection and cocultures of staphylococcus aureus and pseudomonas aeruginosa. Adv Healthc Mater. 2021:e2102539. [CrossRef]

[19] Scherließ R, Janke J. Preparation of poly-lactic-co-glycolic acid nanoparticles in a dry powder formulation for pulmonary antigen delivery. Pharmaceutics. 2021;13(8):1196. [CrossRef]

[20] Wang Q, Chen C, Liu W, He X, Zhou N, Zhang D, Gu H, Li J, Jiang J, Huang W. Levofloxacin loaded mesoporous silica microspheres/nano-hydroxyapatite/polyurethane composite scaffold for the treatment of chronic osteomyelitis with bone defects. Sci Rep. 2017;7(1):41808. [CrossRef]

[21] Schito AM, Schito GC. Levofloxacin, a broad spectrum anti-infective: from Streptococcus pneumoniae to Pseudomonas aeruginosa. J Chemother. 2004;16 Suppl 2:3–7. [CrossRef]

[22] Popa C, Mircheva M, Krämer BK, Berghofen A, Krüger B. Ceftolozane-tazobactam versus levofloxacin in urinary tract infection. Lancet. 2015;386(10000):1241–2. [CrossRef]

[23] Torres A, Liapikou A. Levofloxacin for the treatment of respiratory tract infections. Expert Opin Pharmacother. 2012;13(8):1203–1212. [CrossRef]

[24] Islan GA, Cacicedo ML, Bosio VE, Castro GR. Development and characterization of new enzymatic modified hybrid calcium carbonate microparticles to obtain nano-architectured surfaces for enhanced drug loading. J Colloid Interface Sci. 2015;439:76–87. [CrossRef]

[25] Camargo JA, Sapin A, Daloz D, Maincent P. Ivermectin-loaded microparticles for parenteral sustained release: in vitro characterization and effect of some formulation variables. J Microencapsul. 2010;27(7):609–617. [CrossRef]

[26] Xie X, Yang Y, Chi Q, Li Z, Zhang H, Li Y, Yang Y. Controlled release of dutasteride from biodegradable microspheres: in vitro and in vivo studies. PLoS One. 2014;9(12):e114835–e114835. [CrossRef]

[27] Vora L, V G S, Vavia P. Zero order controlled release delivery of cholecalciferol from injectable biodegradable microsphere: In-vitro characterization and in-vivo pharmacokinetic studies. Eur J Pharm Sci. 2017;107:78–86. [CrossRef]

[28] Zhang H, Pu C, Wang Q, Tan X, Gou J, He H, Zhang Y, Yin T, Wang Y, Tang X. Physicochemical characterization and pharmacokinetics of agomelatine-loaded PLGA microspheres for intramuscular injection. Pharm Res. 2018;36(1):9. [CrossRef]

[29] Wu J, Ding D, Ren G, Xu X, Yin X, Hu Y. Sustained delivery of endostatin improves the efficacy of therapy in Lewis lung cancer model. J Control Release. 2009;134(2):91–97. [CrossRef]

[30] Garg Y, Pathak K. Design and in vitro performance evaluation of purified microparticles of pravastatin sodium for intestinal delivery. AAPS PharmSciTech. 2011;12(2):673–682. [CrossRef]

[31] ICH Guideline Q2(R1), Validation of analytical procedures: text and methodology, November 2005. [CrossRef]

[32] Khoee S, Yaghoobian M. An investigation into the role of surfactants in controlling particle size of polymeric nanocapsules containing penicillin-G in double emulsion. Eur J Med Chem. 2009;44(6):2392–2399. [CrossRef]

[33] Guhagarkar SA, Malshe VC, Devarajan P V. Nanoparticles of polyethylene sebacate: A new biodegradable polymer. AAPS PharmSciTech. 2009;10(3):935. [CrossRef]

[34] Pichot R, Spyropoulos F, Norton IT. O/W emulsions stabilised by both low molecular weight surfactants and colloidal particles: The effect of surfactant type and concentration. J Colloid Interface Sci. 2010;352(1):128–135. [CrossRef]

[35] Anarjan N, Nehdi IA, Tan CP. Influence of astaxanthin, emulsifier and organic phase concentration on physicochemical properties of astaxanthin nanodispersions. Chem Cent J. 2013;7(1):127. [CrossRef]

[36] Mainardes RM, Evangelista RC. PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution. Int J Pharm. 2005;290(1):137–144. [CrossRef]

[37] Chaisri W, Hennink WE, Ampasavate C, Okonogi S. Cephalexin microspheres for dairy mastitis: Effect of preparation method and surfactant type on physicochemical properties of the microspheres. AAPS PharmSciTech. 2010;11(2):945–951. [CrossRef]

[38] Devrim B, Bozkır A. Preparation and evaluation of double-walled microparticles prepared with a modified water-in-oil-in-oil-in-water (w1/o/o/w3) method. J Microencapsul. 2013;30(8):741–754. [CrossRef]

[39] Gaspar MC, Pais AACC, Sousa JJS, Brillaut J, Olivier J-C. Development of levofloxacin-loaded PLGA microspheres of suitable properties for sustained pulmonary release. Int J Pharm. 2019;556:117–124. [CrossRef]

[40] Rojas J, Pinto-Alphandary H, Leo E, Pecquet S, Couvreur P, Fattal E. Optimization of the encapsulation and release of β-lactoglobulin entrapped poly(dl-lactide-co-glycolide) microspheres. Int J Pharm. 1999;183(1):67–71. [CrossRef]

[41] Maa YF, Hsu CC. Effect of primary emulsions on microsphere size and protein-loading in the double emulsion process. J Microencapsul. 1997;14(2):225–241. [CrossRef]

[42] Blanco-Prıeto MJ, Fattal E, Gulik A, Dedieu JC, Roques BP, Couvreur P. Characterization and morphological analysis of a cholecystokinin derivative peptide-loaded poly(lactide-co-glycolide) microspheres prepared by a water-in-oil-in-water emulsion solvent evaporation method. J Control Release. 1997;43(1):81–87. [CrossRef]

[43] Dinarvand R, Moghadam SH, Sheikhi A, Atyabi F. Effect of surfactant HLB and different formulation variables on the properties of poly-D,L-lactide microspheres of naltrexone prepared by double emulsion technique. J Microencapsul. 2005;22(2):139–151. [CrossRef]

[44] Devrim B, Bozkır A, Canefe K. Preparation and evaluation of PLGA microparticles as carrier for the pulmonary delivery of rhIL-2 : I. Effects of some formulation parameters on microparticle characteristics. J Microencapsul. 2011;28(6):582–594. [CrossRef]

[45] Sun Y, Zhu L, Wu T, Cai T, Gunn EM, Yu L. Stability of amorphous pharmaceutical solids: crystal growth mechanisms and effect of polymer additives. AAPS J. 2012;14(3):380–388. [CrossRef]

[46] Ansary RH, Awang MB, Rahman MM. Biodegradable poly(d,l-lactic-co-glycolic acid)-based micro/nanoparticles for sustained release of protein drugs - A review. Trop J Pharm Res. 2014;13(7):1179–1190. [CrossRef]

[47] Wischke C, Schwendeman SP. Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. Int J Pharm. 2008;364(2):298–327. [CrossRef]

[48] Mahboubian A, Hashemein SK, Moghadam S, Atyabi F, Dinarvand R. Preparation and in-vitro evaluation of controlled release PLGA microparticles containing triptoreline. Iran J Pharm Res IJPR. 2010;9(4):369–378. [CrossRef]

[49] Bolourtchian N, Sattari Javid F, Dadashzadeh S. The effect of various surfactants on release behavior of procainamide HCl from ethylcellulose based matrices. Iran J Pharm Res. 2010;4(1):13–19. [CrossRef]

[50] Soares S, Fonte P, Costa A, Andrade J, Seabra V, Ferreira D, Reis S, Sarmento B. Effect of freeze-drying, cryoprotectants and storage conditions on the stability of secondary structure of insulin-loaded solid lipid nanoparticles. Int J Pharm. 2013;456:370-381. [CrossRef]

[51] Oz UC, Küçüktürkmen B, Devrim B, Saka OM, Bozkir A. Development and optimization of alendronate sodium loaded PLGA nanoparticles by central composite design. Macromol Res. 2019;27(9):857–866. [CrossRef]

[52] Gill P, Moghadam TT, Ranjbar B. Differential scanning calorimetry techniques: applications in biology and nanoscience. J Biomol Tech. 2010;21(4):167–193. [CrossRef]

[53] Gorman EM, Samas B, Munson EJ. Understanding the dehydration of levofloxacin hemihydrate. J Pharm Sci. 2012;101(9):3319–3330. [CrossRef]

[54] Akdag Cayli Y, Sahin S, Buttini F, Balducci AG, Montanari S, Vural I, Oner L. Dry powders for the inhalation of ciprofloxacin or levofloxacin combined with a mucolytic agent for cystic fibrosis patients. Drug Dev Ind Pharm. 2017;43(8):1378–1389. [CrossRef]

[55] Dillen K, Vandervoort J, Van den Mooter G, Verheyden L, Ludwig A. Factorial design, physicochemical characterisation and activity of ciprofloxacin-PLGA nanoparticles. Int J Pharm. 2004;275(1):171–187. [CrossRef]

[56] Sant S, Nadeau V, Hildgen P. Effect of porosity on the release kinetics of propafenone-loaded PEG-g-PLA nanoparticles. J Control Release. 2005;107(2):203–214. [CrossRef]

[57] Jalvandi J, White M, Gao Y, Truong YB, Padhye R, Kyratzis IL. Polyvinyl alcohol composite nanofibres containing conjugated levofloxacin-chitosan for controlled drug release. Mater Sci Eng C. 2017;73:440–446. [CrossRef]

[58] Jamrógiewicz M, Łukasiak J. Short term monitor of photodegradation processes in ranitidine hydrochloride observed by FTIR and ATR-FTIR. J Food Drug Anal. 2009;17(5):342–347. [CrossRef]

[59] Jain H, Bairagi A, Srivastava S, Singh SB, Mehra NK. Recent advances in the development of microparticles for pulmonary administration. Drug Discov Today. 2020 Oct;25(10):1865-1872. [CrossRef]

[60] Yang MY, Chan JGY, Chan H-K. Pulmonary drug delivery by powder aerosols. J Control Release. 2014;193:228–240. [CrossRef]

[61] Lee J-H, Park TG, Choi H-K. Effect of formulation and processing variables on the characteristics of microspheres for water-soluble drugs prepared by w/o/o double emulsion solvent diffusion method. Int J Pharm. 2000;196(1):75–83. [CrossRef]

[62] Mahmoud BS, McConville C. Development and optimization of irinotecan-loaded PCl nanoparticles and their cytotoxicity against primary high-grade glioma cells. Pharmaceutics. 2021;13(4):541. [CrossRef]

[63] Watts AB, Cline AM, Saad AR, Johnson SB, Peters JI, Williams RO. Characterization and pharmacokinetic analysis of tacrolimus dispersion for nebulization in a lung transplanted rodent model. Int J Pharm. 2010;384(1):46–52. [CrossRef]

[64] Eskandar F, Lejeune M, Edge S. Low powder mass filling of dry powder inhalation formulations. Drug Dev Ind Pharm. 2011;37(1):24–32. [CrossRef]

[65] Jalalipour M, Gilani K, Tajerzadeh H, Najafabadi AR, Barghi M. Characterization and aerodynamic evaluation of spray dried recombinant human growth hormone using protein stabilizing agents. Int J Pharm. 2008;352(1):209–216. [CrossRef]

[66] Ameeduzzafar, Imam SS, Abbas Bukhari SN, Ahmad J, Ali A. Formulation and optimization of levofloxacin loaded chitosan nanoparticle for ocular delivery: In-vitro characterization, ocular tolerance and antibacterial activity. Int J Biol Macromol. 2018;108:650–659. [CrossRef]