Formulation and In Vitro Characterization of Polymeric Nanoparticles Designed for Oral Delivery of Levofloxacin Hemihydrate

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Abstract
In the present study, levofloxacin hemihydrate (Levo-h) was successfully incorporated into cationic Eudragit® RL 100 (ERL) nanoparticles by spray-drying method aiming oral application. Particle size and zeta potential measurements, entrapment efficiency, morphological, thermal, FTIR and NMR analyses and Levo-h quantification by UV analyses were performed for characterizing the formulations prepared. Release pattern of Levo-h from the nanoparticles was determined using a dialysis tube. Following successful incorporation of Levo-h into the polymeric nanoparticles, ERL-Levo-h (10:1) was selected for further studies considering smaller particle size with narrow size distribution, cationic zeta potential, possible drug polymer interaction indicated by thermal, FTIR and NMR analyses and prolonged release behavior. In vitro characterization results showed that Eudragit® RL 100 nanoparticles could enhance bioavailability, decrease Levo-h inactivation and also reduce the potential of drug resistance.

Keywords: Levofloxacin hemihydrate, Eudragit, polymeric nanoparticle, spray-drying

1. Introduction
Levofloxacin, a third generation fluoroquinolone antibacterial agent, has a broad spectrum of activity against gram positive and gram negative bacterii (Verma and Doshi, 2014). Fluoroquinolone derivatives are used mainly for the treatment of urinary tract, respiratory tract, skin and soft tissue infections. Levofloxacin hemihydrate (Levo-h) is rapidly and completely absorbed after oral administration. The mean terminal plasma elimination half-life of levofloxacin ranges from approximately 6 to 8 h following single or multiple doses given orally or intravenously (Diren and Zeynep, 2007). It requires frequent dosing to maintain therapeutic effect due to its short biological half-life and highly varying pharmaceutical concentrations in blood. Therefore, it may be a good idea to formulate Levo-h loaded nanoparticles as a controlled release drug delivery system to obtain longer blood circulation than its half-life to improve bioavailability, reduce dose frequency, toxicity and also to improve patient compliance (Hasan et al., 2015).

Many efforts towards developing levofloxacin sustained delivery systems by encapsulating (El-Zahaby et al., 2014), forming filament (Mack et al., 2009), microsphere (Balaji et al., 2015) and nanoparticle (Hasan et
al., 2015) were made during the last few years. Since nanoparticles have decreased particle size, increased surface area, enhanced reactivity, promoted drug dissolution, reformed targeting, reduced toxicity and improved sustained-release efficacy, they can offer numerous advantages over the conventional dosage forms. Therefore nanoparticles attracted considerable attention due to their advantageous properties (Guan et al., 2011).

Surface charges of nanoparticles have significant impact on interaction with cells and also on their uptake. Positively charged nanoparticles seem to allow higher extent of internalization apparently as a result of the ionic interactions established between positively charged particles and negatively charged membranes (Foged et al., 2005).

Eudragit® RL 100 polymer is a copolymer, poly(ethylacrylate-methylmethacrylate), containing 8.8% - 12% quaternary ammonium groups (Singh and Pai, 2016). Presence of quaternary ammonium group renders positive charge to the polymer by which it can interact with anionic drugs and GIT mucus (Srinivas and Sumapriya, 2014). It is a suitable inert carrier for drug delivery due to its capability to form nanodispersion with small particle size, positive surface charge, good stability and biocompatibility (Srinivas and Pragna, 2012). It also prolongs residence time of drugs resulting in sustained drug release (Singh and Pai, 2016).

Spray-drying represents a single-step, continuous and scalable process dedicated for converting liquid streams (solutions, emulsions, suspensions, slurries, pastes or even melts) into dry, free-flowing powders which enables the production of particles with controlled size and morphological aspects. Spray-drying process also allows the encapsulation of active agents thus opening a wide spectrum of opportunities in the field of particle engineering in pharmaceutical, materials and food science (Re, 2006). In spray-drying technology it is possible to produce powders with high formulation yield and particles in the submicron range with very narrow distributions (Li et al., 2010). Spray-drying technology was utilized in this study for the formulation of cationic nanoparticles using advantages of the method.

The aim of the study was to formulate positively charged Levo-h loaded nanoparticles at submicron level with high entrapment efficiency and prolonged effect for optimizing plasma drug concentrations that improves bioavailability, reduces dose frequency and protects its inactivation.

2. Materials and Methods

2.1. Materials

Levofloxacin hemihydrate (Lev-h) was a gift from Neutec (İstanbul, Turkey). Eudragit® RL 100 was purchased from Röhm Pharma Polymers (Germany). Methanol was obtained from Sigma-Aldrich (Israel). Potassium dihydrogen phosphate and sodium hydroxide were purchased from Merck, Germany. All other reagents used were of analytical grade.

2.2. Preparation of Nanoparticles

Spray-drying method was used for the preparation of nanoparticles. Initially, accurately weighed Eudragit® RL 100 was dissolved in methanol (96 mL) at room temperature. The active agent Levo-h was solubilized in methanol-water (50:50, v/v) (8 mL). Levo-h solution was added to the polymer solution under mild agitation (350 rpm). Final transparent solution was then spray-dried using a Nano Spray Dryer (B-90, BUCHI Labortechnik AG, Switzerland) with an inlet temperature of 120°C ± 1°C and outlet temperature 50°C ± 5°C. Yellowish dry powders were obtained and kept in tightly closed and colored vials at the refrigerator until being analyzed. Placebo formulations were prepared as described above without the addition of Levo-h.

Compositions of polymeric nanoparticles prepared are given in Table 1.
Table 1. Compositions of nanoparticles prepared. (ERL: Eudragit® RL 100, Levo-h: Levofloxacin hemihydrate)

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>ERL Ratio</th>
<th>Levo-h Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERL-Levo-h 1</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>ERL-Levo-h 2</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>ERL-Levo-h 3</td>
<td>10</td>
<td>0.25 g</td>
</tr>
<tr>
<td>ERL Placebo</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3. Characterization of Nanoparticles

2.3.1. Morphology

Structural and morphological properties of the nanoparticles were investigated using scanning electron microscopy (SEM) (FESEM SUPRATN 50 VP ZEISS, Germany). Samples were coated with gold under argon to avoid any charging under the electron beam.

2.3.2. Particle size and zeta potential

Particle size and zeta potential measurements were performed on freshly prepared samples using Malvern Nano ZS (Zetasizer Nano Series, Worcestershire, UK). Samples of all nanoparticles were dispersed in double-distilled water (adjusted to a constant conductivity of 50 \( \mu \text{S} \cdot \text{cm}^{-1} \) using 0.9 % NaCl) just prior to analyses. All analyses were repeated in triplicate.

2.3.3. Differential scanning calorimetry (DSC)

Crystallinity and any structure changes of Lev-h and the polymer were analyzed using differential scanning calorimetry (DSC) (DSC; DSC-60, Shimadzu Scientific Instruments, Columbia, MI, USA). DSC analyses were carried out under nitrogen at a flow rate of 50 \( \text{mL} \cdot \text{min}^{-1} \) at 50-300°C with an increase rate of 10°Cmin\(^{-1}\).

2.3.4. Fourier transform infrared spectrophotometry (FT-IR)

Fourier transform infrared spectrophotometric (FTIR) analysis spectra were recorded using Shimadzu IR Prestige-21 (Japan) and the formulations were analyzed at the wavelength range of 4000-500 cm\(^{-1}\). FT-IR spectra of pure LEVO and the polymer were used as references.

2.3.5. Nuclear magnetic resonance spectroscopy (NMR)

NMR analyses were performed on Fourier 300 FT-NMR spectrometer (Brucker, USA) using deuterated dimethyl sulfoxide ((CD\(_3\))\(_2\)S=O) as the solvent. Spectra of pure Levo-h and pure HNT were also analyzed and used as references.

2.4. Drug loading and entrapment efficiency

For the determination of Levo-h entrapment efficiency, accurately weighed nanoparticles (5mg) were dispersed in bidistilled water (2 mL) via ultrasonic bath at room temperature and centrifuged at 11000 rpm for 15 min. Then the amount of Levo-h remaining on polymeric nanoparticle surface (Levo-h\(_s\)) was measured by UV analysis (Shimadzu UV-1280 (Kyoto, Japan) at 288 nm. Some samples were submitted to a second centrifugation procedure. Pellets obtained after centrifugation was dissolved in methanol (2mL) and the amount of Levo-h encapsulated (Levo-h\(_e\)) in polymeric nanoparticles was measured by UV spectrophotometer. The experiment was repeated in triplicate. Encapsulation efficiency (EE) (Equation 1) was calculated using the equations below:
Drug loading (%) = \[\left(\text{Levo-h}_c + (\text{Levo-h}_s) \times \text{particle weight}^{-1}\right) \times 100\]  
Equation 1

\[
\text{EE} (%) = \left[\left(\text{Levo-h}_c\right) \times (\text{Levo-h}_s + \text{Levo-h}_c) \times 100\right]
\]
Equation 2

2.5. In vitro release

In vitro release of Levo-h from polymeric nanoparticles was investigated using dialysis bag method over 48hr. Drug loaded nanoparticles equivalent to 2 mg were suspended in 1 mL of phosphate buffer (pH 6.8) (donor medium) in a dialysis bag (with a molecular weight cut off 12-14 kDa, Sigma) and was dialyzed against 50 mL of phosphate buffer (pH 6.8) (receptor medium). Medium was continuously stirred at 50 rpm and maintained at a temperature of 37°C ± 1°C. The receptor compartment was closed to prevent evaporation of the dissolution medium. Samples were withdrawn at regular time intervals and the same volume was replaced by fresh dissolution medium. Levo-h concentration was measured using UV- spectrophotometer at 288 nm against blank.

3. Results and Discussion

3.1. Characterization of nanoparticles

3.1.1. Morphology

SEM images of formulations showed nearly spherical shapes while some of the spheres reminded the collapsed balloons with smooth surfaces (Figure 1).

Figure 1. SEM images of nanoparticles prepared. [a: ERL-Levo-h 1, b: ERL-Levo-h 2, c: ERL-Levo-h 1, x 50000].
3.1.2. Particle size and zeta potential

Particle size is one of the important physical properties of colloidal systems. Particle size distribution of the formulation is especially significant in the physical stability and activity of colloidal systems (Takka et al., 2007). It was also found that the size of nanoparticles plays a key role in their adhesion to and interaction with biological cells (Wina and Feng, 2005). The mean particle size of Levo-h loaded nanoparticles of all formulations ranged from 232 to 427 nm with a relative monodisperse distribution (Table 2). Decrease in the amount of Levo-h in formulations was in parallel with the relative decrease in average particle size. The acceptable value for polydispersity index (PI) is 0.05-0.7; values greater than 0.7 indicate very broad size distribution and probably no suitability for dynamic light scattering technique (Hasan et al., 2015). As shown in Table 2, acceptable values for PI were obtained for all batches.

Zeta potential of nanoparticles is commonly used to characterize the surface property of nanoparticles. Nanoparticles tend to be electrically charged on their surfaces in aqueous electrolytes by adsorbing ions onto surfaces and nanoparticles tend to aggregate when electrostatic force is lower than Van Der Walls force (Man et al., 2014). Nanoparticles with a zeta potential above 30 mV were shown to be stable in suspensions as the surface charge prevents aggregation of the particles (Balaji et al., 2015). Results showed that zeta potentials measured were 39.23 ± 0.18 mV, 38.43 ± 0.15 mV and 41.33 ± 0.47 mV for ERL-Levo-h 1, ERL-Levo-h 2 and ERL-Levo-h 3 formulations, respectively, indicating good physical stability (Table 2). Cationic property of nanoparticles was determined due to the predominant effect of positively charged quaternary ammonium groups in Eudragit® structure (Hasan et al., 2015). As suggested by Ubricha et al. (2005), positively-charged polymers such as chitosan and Eudragit® RS and RL may interact with the negatively charged mucus and open up the tight junctions of epithelial cells to allow the paracellular transport pathway resulting in an increase in bioavailability (Ubricha et al., 2005).

Table 2. Mean particle size, polydispersity index, zeta potential and Levo-h content of nanoparticles prepared. (Mean± SE)(ERL: Eudragit® RL 100, Levo-h: Levofloxacin hemihydrate, PI: Polydispersity index, EE: Encapsulation Efficiency, SE: Standard Error)

<table>
<thead>
<tr>
<th>Code</th>
<th>Particle Size (nm)</th>
<th>PI</th>
<th>Zeta Potential (mV)</th>
<th>EE (%)</th>
<th>Drug loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERL-Levo-h 1</td>
<td>226.70 ± 12.88</td>
<td>0.41 ± 0.05</td>
<td>39.23 ± 0.18</td>
<td>11.33 ± 1.34</td>
<td>8.70 ± 0.48</td>
</tr>
<tr>
<td>ERL-Levo-h 2</td>
<td>269.43 ± 21.16</td>
<td>0.32 ± 0.06</td>
<td>38.43 ± 0.15</td>
<td>9.07 ± 0.63</td>
<td>4.69 ± 0.51</td>
</tr>
<tr>
<td>ERL-Levo-h 3</td>
<td>232.40 ± 15.56</td>
<td>0.36 ± 0.04</td>
<td>41.33 ± 0.47</td>
<td>7.73 ± 0.86</td>
<td>2.71 ± 0.07</td>
</tr>
<tr>
<td>ERL Placebo</td>
<td>268.97 ± 27.23</td>
<td>0.43 ± 0.04</td>
<td>42.90 ± 0.67</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

3.1.3. DSC analysis

Thermodynamic variations related to morphological changes during and after formulation steps can be detected by DSC. Figure 2 displays the thermal behavior of Levo-h and the nanoparticles prepared. According to the DSC results, placebo formulation was determined to be in amorphous state revealing no sharp peaks in the thermogram. Pure Levo-h exhibited an endothermic peak at 229°C due to melting of the γ form, an endothermic peak at 233°C due to the melting form of the β form and an endothermic peak at
235°C due to the melting of the $\alpha$ form (El-Zahaby et al., 2014). However, the melting peak of Levo-h was not seen in the DSC thermograms of the formulations showing that Levo-h was molecularly dispersed in the polymeric structure (Figure 2).

![DSC thermograms of pure Levo-h and nanoparticles prepared.](image)

**Figure 2. DSC thermograms of pure Levo-h and nanoparticles prepared.**

### 3.1.4. FTIR analysis

FTIR measurements were conducted to assess possible microstructural changes in the polymeric structure (Saito and Iwata, 2012). Levo-h FTIR peaks demonstrated characteristics absorption peaks for the –OH group of the –COOH moiety at around 3261.3 cm$^{-1}$ and –C=O peak at 1724.1 cm$^{-1}$. Aromatic C-H peaks were also observed in the range 2900-3000 cm$^{-1}$ (El-Zahaby et al., 2014). Absorption bands at 2950-3000 cm$^{-1}$ in the FTIR spectrum of placebo formulation were assigned to C-H group vibration. The bands observed at 1728.22 cm$^{-1}$ and 1147.65 cm$^{-1}$ were a result of –C=O group and –C-O band of the ester moiety, respectively (Eudragit®, 2016). Distinctive peaks of Levo-h were not seen in the spectra of formulations indicating the molecular dispersion of Levo-h in the polymeric structure which was supported also by DSC.

### 3.1.5. NMR analysis

$^1$H NMR analysis was applied effectively to characterize the form of Levo-h within the polymeric matrix, molecular mobility and molecular interactions between drug and polymer (Li et al., 2008). All principal peaks were observed in the spectrum of Levo-h. Peaks in 7-8 ppm interval belong to aromatic C-H peaks, 1-5 ppm interval belong to –CH and –CH$_2$ groups and peaks at 2.38 pm and 4.72 ppm belong to –CH$_3$ in the spectrum of Levo-h. Similar spectra were obtained for ERL-Levo-h 1 and ERL-Levo-h 2, some of characteristic peaks of Levo-h were found also in spectra of nanoparticle formulations indicating successful incorporation of drug into the nanoparticles. However, Levo-h signals could not be detected for ERL-Levo-h 3 formulation. This may be attributed to the low concentration of Levo-h (Jenning et al., 2000).
3.2. Drug loading and encapsulation efficiency
Drug loading (%) and EE (%) were evaluated according to Equations [1] and [2], respectively. Levo-h EE(%) was determined to be 11.33 ± 1.34, 9.07 ± 0.63, 7.73 ± 0.86 (% ± SE) while drug loading was 8.70 ± 0.48, 4.69 ± 0.51 and 2.71 ± 0.07 (% ± SE) for ERL-Levo-h 1, ERL-Levo-h 2 and ERL-Levo-h 3, respectively (Table 2). It is evident from the data that EE (%) was affected by the drug-polymer ratio. The results revealed that the EE (%) of all formulations was increased with increasing concentration of the active agent in the formulations.

3.3. In vitro release study
Release profiles obtained for Eudragit® RL 100 nanoparticles in pH 6.8 phosphate buffer are given in Figure 5. In vitro release profiles exhibited an initial rapid release in 30 minutes which can be attributed to the fraction of drug which was weakly encapsulated or adsorbed onto the surface of nanoparticles. This initial rapid release was followed by an extended release owing to the polymeric matrix. An initial rapid release pattern is beneficial in terms of antibacterial activity as it helps to achieve the therapeutic concentration of the drug immediately prior to slow release to maintain and sustain the drug effect (Mudgil and Pawar, 2013). Levo-h release from nanoparticles was much lower than its pure form showing time dependent release manner in pH 6.8 phosphate buffer. Significant low Levo-h release was determined from ERL-Levo-h 1 formulation compared to other formulations which may be attributed to the relatively high EE of ERL-Levo-h 1. It can be said that as the EE increased, drug release was retarded.

Figure 3. FTIR spectra of pure Levo-h and nanoparticles prepared.
Figure 4. NMR spectra of pure Levo-h and nanoparticles prepared.

Figure 5. *In vitro* release of Levo-h from polymeric nanoparticles prepared (n=6, Mean ± SE).
4. Conclusion
In this study, cationic nanoparticles were prepared by spray-drying method for potential oral delivery of Levo-h. Attempt was made to prepare nanoparticles with prolonged release of Levo-h by the spray-drying technique using Eudragit® RL 100 as the polymer and evaluated with particle size and zeta potential measurements, entrapment efficiency, thermal behavior, FTIR, NMR analyses and in vitro drug release test. Among the formulations prepared, ERL-Levo-h 1 showed lower particle size, narrow size distribution, relatively high entrapment efficiency and prolonged release pattern. DSC, FTIR and NMR studies also confirmed successful incorporation of Levo-h into the nanoparticles. According to in vitro release studies, Levo-h nanoparticles showed burst release in the first 30 minutes after application followed by sustained release for 24 hr.

In vitro studies showed that cationic Eudragit® RL 100 nanoparticles containing Levo-h is a promising formulation for optimizing plasma drug concentration which can improve bioavailability, reduce dose frequency and protect from inactivation, thus also reducing the potential drug resistance.

Declaration of Interest
The authors report no conflict of interest. The authors are responsible for the content and writing this article.

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