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Development and physical characterization of chloramphenicol loaded biodegradable nanoparticles for prolonged release

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The objectives of our study were to prepare a biodegradable nanoparticulate system of chloramphenicol (CHL) and to evaluate its ability to prolong *in vitro* release of CHL compared to free drug suspension (FDS). CHL-loaded polylactide-co-glycolide nanoparticles (CHL-PLGA-NPs) were prepared by an emulsion/solvent evaporation method using ethyl acetate and polyvinyl alcohol. CHL-PLGA-NPs were characterized by particle size, zeta potential, infrared spectra, drug entrapment efficiency and *in vitro* release kinetics measurement. Sonication was done with an ultrasound pulse sonicator at 70 W, 30 kHz for 60 s to produce stable NPs of mean size range from 277 nm to 433 nm. Drug to polymer ratio (D : P) was selected as formulation variable and significantly influenced entrapment efficiency (~30% to 66%) and release ($p < 0.05$). Entrapment of CHL in biodegradable NPs significantly prolonged drug release compared to FDS and thus implies potential antibiotic delivery system for ocular application.

1. Introduction

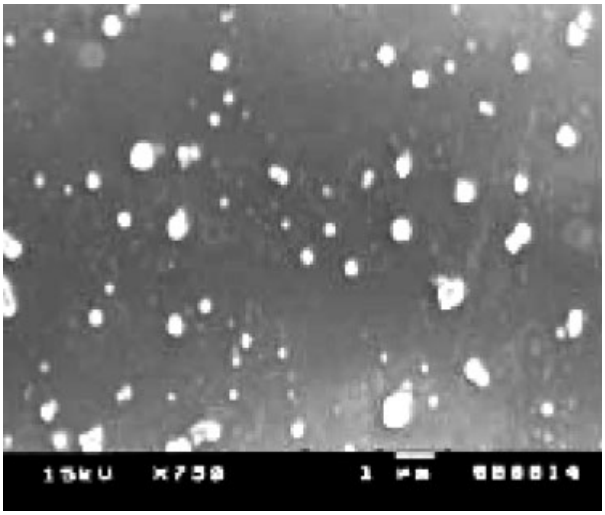
Entrapment of antibiotics in nanoparticles (NPs) has resulted in higher efficiency, diminished toxicity, advantages related to targetability and controlled release compared to the free antimicrobials. In addition to parenteral and oral administration, biodegradable polymeric NPs have shown promising results as ophthalmic drug carriers (Bucolo et al. 2002; Pignatello et al. 2002; Bonduelle et al. 1996). Most commonly used polymers for ocular NPs are poly (alkylcyanoacrylates), poly-ε-caprolactone and polylactide-co-glycolide (PLGA), which are biodegradable and undergo hydrolysis in tears (Ding 1998). NPs, because of their polymeric nature, present some important advantages over other colloidal carriers for ophthalmic applications such as high storage stability, controlled release of the encapsulated drug, prolonged residence time in precorneal area, and increased ocular availability, particularly beneficial in case of ocular inflammation and infection (e.g. acute bacterial conjunctivitis). The mechanism underlying the phenomena of enhanced ocular availability of drug entrapped in PLGA NPs is related to the absorptive type endocytosis (Qaddoumi et al. 2004).

Acute bacterial conjunctivitis is a prevalent infection in the population. Ocular antibiotic treatment is recommended to eradicate the pathogen and reduce symptom duration of acute bacterial conjunctivitis. Topically applied chloramphenicol (CHL) in the form of eye drops has been the most frequently recommended treatment of this indication. CHL has shown good activity to *Staphylococcus aureus* in cornea and conjunctiva (Chalita et al. 2004) and methicillin-resistant *Staphylococcus aureus* (MRSA) ocular surface infections (Fukuda et al. 2002). The recommended dosage is one-drop every 1–2 h for three days, thereafter every 4–6 h (Laerum et al. 1994). CHL has half-life of 3–5 h, and is therefore a suitable candidate for prolonged ocular drug delivery systems with the rationality of reducing the frequency of dosing.

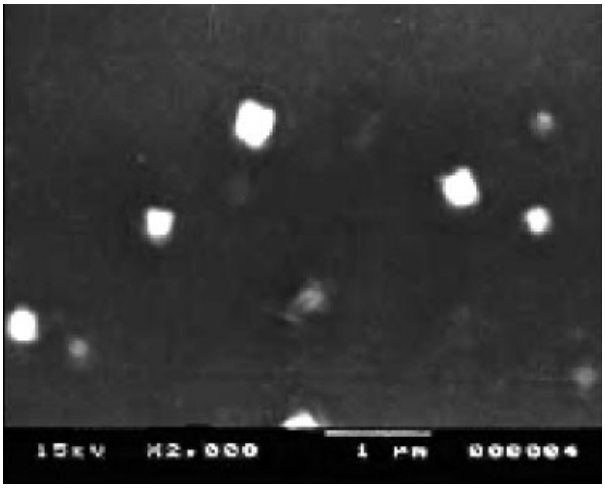
We have prepared CHL-PLGA-NPs by the emulsion-solvent evaporation technique utilizing ethyl acetate as disperse solvent and polyvinyl alcohol as colloid stabilizer. Ethyl acetate is environmentally and biologically safer than methylene chloride (Birnbaum et al. 2000) and considered a better solvent in terms of producing reduced particle size (Mainardes et al. 2005). Particle size, morphology, entrapment efficiency, drug-polymer interaction and release kinetics of CHL-PLGA-NPs were evaluated. The influence of drug to polymer ratio on formulation parameters including particle size, zeta potential, entrapment efficiency, *in vitro* release and stability were investigated. *In vitro* release performances of different formulations have also been compared to release profile of free drug suspension (FDS).

2. Investigations, results and discussion

The emulsion-solvent evaporation method is commonly used for preparation of NPs to reduce particle size, and increase entrapment efficiency of lipophilic drugs. NPs prepared by emulsion-solvent evaporation method exhibited nearly spherical shape with a smooth surface (Fig. 1a, b and c), which was independent of drug-polymer ratio. But, the mean size range as calculated from electron microscopic measurements exhibited to be wide (200 nm–440 nm). Few aggregations of particles can be seen in



(a)



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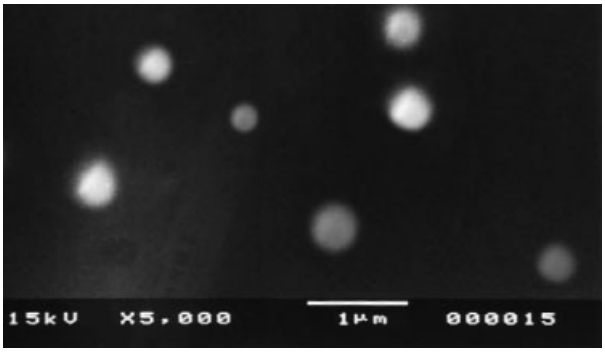
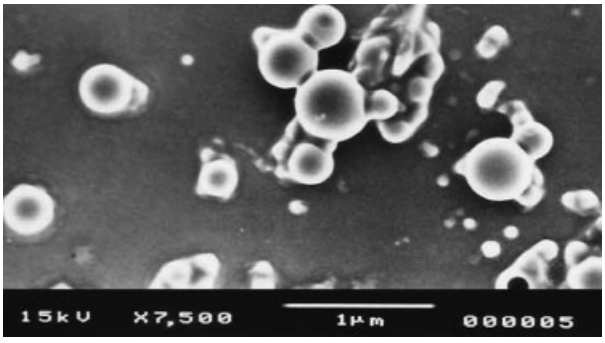


(c)



(d)

Fig. 1: Scanning electron micrograph of CHL-PLGA-NPs (75 : 25) (D : P = 3 : 10) [A: lower magnification, B: higher magnification] and C: Blank NPs, D: Blank NPs showing aggregation]

electron microscopy although they are not larger than 1000 nm (Fig. 1d). The mean diameter of NPs varied from 277 nm–423 nm, when the theoretical amount of CHL was increased from 10–30% in relation to polymer mass (Table 1). This can be explained by the fact that a greater amount of drug results in a more viscous dispersed phase, making difficult the dispersion between phases and originating larger particles. During emulsification process, the lower the viscosity of the dispersed phase, the smaller the particles mean diameter. A more viscous organic phase provides a higher mass transfer resistance, the diffusion of polymer-solvent phase into the external phase is reduced and larger particles are formed. Moreover, the addition of organic phase to aqueous phase under magnetic stirring is critical. Rapid addition under vigorous stirring caused formation of fibre like agglomerates, which eventually separated from aqueous phase before forming globules. This problem is generally prevalent with the use of ethyl acetate because of its partial miscibility with water and rapid evaporative nature. Slow stirring with continuous dropwise addition of organic phase to aqueous phase formed stable microdroplets (visible fine droplets of organic phase dispersed in aqueous medium), which was further sonicated for successful formation of NPs.

The zeta potential values of blank NPs and CHL-PLGA-NPs measured in double distilled water (Table 1). All NPs exhibited strongly negative zeta potential values in water since all PLGA NPs contain free carboxyl end groups on particle surface. Compared to blank NPs, the presence of CHL into CHL-PLGA-NPs did not influence the zeta potential in a significant way. The progressive zeta potential reduction was shown among three formulations with changing drug-to-polymer ratio. This phenomenon can be correlated with particle size of NPs. The higher average particle size of NPs, lowered the electrophoretic mobility of particles, thus decreased zeta potential value compared to smaller particles.

The lower entrapment efficiencies obtained with the smaller particles (mean diameter 277 nm) could be explained by the larger surface area of the droplets during the emulsification step and, therefore, a more direct contact between internal and external phases occurs, resulting in a higher drug loss by diffusion towards the external medium. Entrapment efficiency of CHL-PLGA-NPs increased from about 30% to 66% with increase in mean diameter of NPs from 277 nm to 423 nm (Fig. 2). The polymer solution rapidly precipitated into fiber-like agglomerates upon addition to the external aqueous phase, prior to the dispersion of the polymer solution into droplets. This is due to partial miscibility of ethyl acetate with water (10%

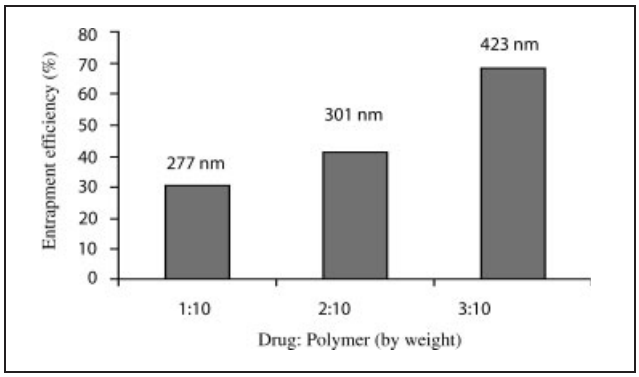


Fig. 2: Entrapment efficiency (%, mean value) of chloramphenicol in PLGA NPs as a function of drug-to-polymer ratio and mean diameter (nm)

Table 1: Influence of drug: polymer ratio (D : P) on physicochemical characteristics, entrapment efficiency and drug loading of CHL-PLGA-NPs

D : P	Initial		After 3 months at 4 ± 2 °C			Entrapment efficiency (%)	Drug loading (μg/mg polymer)
	Size (nm)	Zeta potential (mV)	Size (nm)	Zeta potential (mV)	Drug leakage (%)		
Blank	269 ± 11	−27.5 ± 1.7	289 ± 17	−28.1 ± 2.6			
1 : 10	277 ± 20	−24.6 ± 2.9	293 ± 22	−24.3 ± 1.6	4.54 ± 1.23	30.49 ± 1.5	60.9 ± 1.1
2 : 10	301 ± 21	−23.1 ± 1.8	284 ± 13	−21.6 ± 1.4	4.81 ± 1.16	41.45 ± 3.3	82.9 ± 0.9
3 : 10	423 ± 26	−17.4 ± 1.1	456 ± 26	−15.8 ± 2.8	3.56 ± 1.34	68.44 ± 2.5	205.3 ± 1.0

v/v at 25 °C). Rapid partitioning of ethyl acetate in the external phase caused polymer phase separation.

In the present work, drug polymer interaction was assessed by the FTIR for pure PLGA, pure CHL and CHL-PLGA-NPs samples (Fig. 3). No significant differences on shape and position of the absorption peaks could be clearly observed throughout the batches of the samples. CHL sample showed the main peaks contributed by the functional groups of molecule such as carbonyl −C=O stretching vibrations (1686 cm^{-1}), −N−H stretching (3263 cm^{-1}), −O−H stretching vibrations (3349 cm^{-1}), $-NO_2$ asym. stretching (1521 cm^{-1}). PLGA sample showed peaks such as −CH, $-CH_2$, $-CH_3$ stretching (2850–3000 cm^{-1}), carbonyl −C=O stretching vibrations (1700–1800 cm^{-1}), C−O stretching (1050–1250 cm^{-1}) and −OH stretching vibrations (3200–3500 cm^{-1}) which were broad. CHL-PLGA-NPs showed peaks resulting from simple superposition of their separated components infrared curves. The spectral analysis indicated the specific functional groups of polymeric material on the surface of NPs are almost the same chemical characteristics. It can be concluded that no strong chemical interaction occurred between drug and polymer.

In vitro drug release from CHL-PLGA-NPs was assessed in phosphate buffer (pH 7.4) by dialysis experiments (Fig. 4), shows the in vitro release curves of FDS and three different compositions of CHL-PLGA-NPs. Evidently, CHL showed a slower and prolonged dissolution profile for 10 h from NPs than FDS. While about 100% CHL released from FDS in only 3 h, 27.44, 42.43 and 65.77% of CHL were released after 10 h from batches containing drug-to-polymer ratio 1 : 10, 2 : 10, 3 : 10 respectively. For all NP formulations, the release rate was related to the drug-to-polymer ratio. The batch with a drug polymer ratio of 1 : 10 showed the slowest release rate. Different release profiles can be correlated with mean diameter and entrapment efficiency. Formulation (D : P = 3 : 10) with highest CHL entrapment (66%) and biggest mean diameter (423 nm) showed the highest drug release rate and cumulative drug release (65.77%) while the formulation (D : P = 1 : 10) with the lowest CHL entrapment (30.49%) and smallest mean diameter (277 nm) showed only 27.44% cumulative drug release. An important phenomenon observed is that, as the amount of drug present in NPs gets higher, drug release occurred more quickly and the particles with lowest CHL entrapment exhibited slower release profile. Such a character would suggest that more homogenous and finer dispersion of drug molecules at lower drug concentration in polymer matrix retarded diffusion of drug molecules in release media while different drug distribution at higher drug concentration in polymer matrix during NPs formation resulted in higher drug release.

In general, all three CHL-PLGA-NPs formulations showed prolonged release; no burst effect could be observed. Correlation coefficients (R^2) and diffusion exponents (n) of release profiles showed that drug release best fitted the Higuchi model (Table 2). Such a behavior would suggest that a diffusive (matrix-type) pattern of release took place (Giannavola et al. 2003). The diffusion of the drug, erosion and swelling of polymer matrix and the degradation of polymer are the main mechanisms of drug release. Since the degradation of hydrophobic PLGA (75 : 25) is slow, the release of CHL from NPs would mainly depend on drug diffusion and matrix erosion. Two formulations showed a diffusion exponent n > 5 indicating non-fickian/anomalous transport mechanism. Again, drug diffusion and matrix erosion process depend on size, hardness and

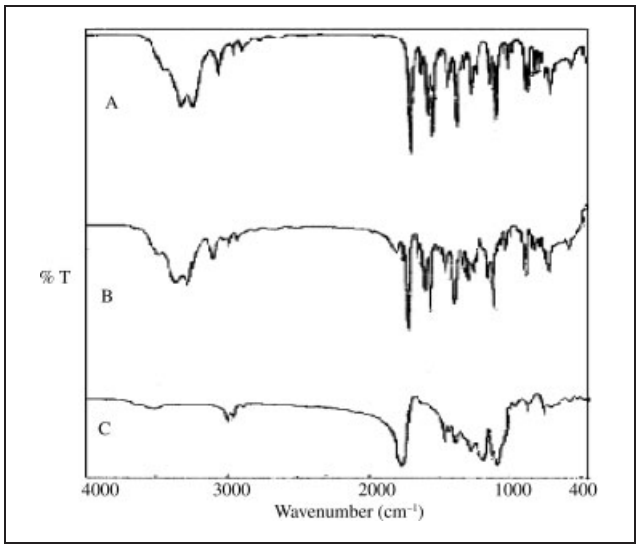


Fig. 3: Infrared spectra of: (A) pure CHL; (B) CHL-PLGA- NPs; (C) pure PLGA

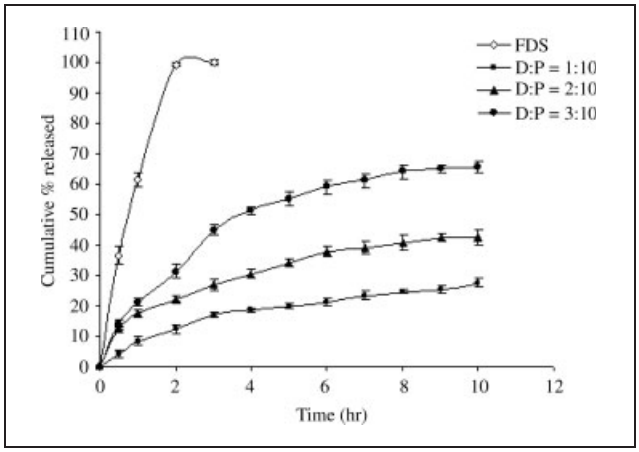


Fig. 4: In vitro release of chloramphenicol (CHL) from free drug suspension (FDS) and CHL-PLGA-NPs suspension having different drug to polymer ratio (D : P)

Table 2: Correlation coefficients (R^2) and diffusion exponent (n), mean ± SD calculated after fitting the release profiles into different kinetic models

Drug: Polymer (w/w)	Zero order R^2	First order R^2	Higuchi R^2	Hixon-Crowell R^2	Korsemeyer-Peppas R^2	n	Model of best fit
1 : 10	0.8994 ± 0.065	0.9232 ± 0.021	0.9892 ± 0.0458	0.5884 ± 1.0154	0.9704	0.5823 ± 0.037	Higuchi
2 : 10	0.8691 ± 0.015	0.9159 ± 0.0125	0.9874 ± 0.023	0.5688 ± 0.9185	0.996	0.4191 ± 0.025	Higuchi
3 : 10	0.8543 ± 0.027	0.9283 ± 0.0229	0.9735 ± 0.052	0.5489 ± 01.9023	0.9944	0.614 ± 0.014	Higuchi

porosity of NPs. SEM examination indicated that spherical, smooth surface of all NPs, which supported slow release of drug. From plot, it was observed that there were significant changes in drug release with decreasing amount of polymer in formulation. Increasing polymer proportion in the formulation decreased drug release from CHL-PLGA-NPs.

After 3 months storage at 4 °C, Zeta potential of CHL-PLGA-NPs did not change significantly. This indicated that no significant drug leakage (<10%, Table 1) from NPs to the aqueous environment occurred. If significant amount of drug escapes from CHL-PLGA-NPs, there would be different amount of surface charge causing significant change in zeta potential value. The released drug from NPs may act as initial booster dose while drug entrapped inside NPs may serve as subsequent maintenance dose. During storage, three formulations of CHL-PLGA-NPs formed loose sediment of particles, which could be easily redispersed by manual shaking.

3. Experimental

3.1. Chemicals

The biodegradable polymer studied was poly(D, L-lactide-co-glycolide), known as PLGA, with a copolymer ratio of D, L-lactide to glycolide of 75 : 25 (molecular weight 15000 Da, free acid 0.2%, inherent viscosity 0.166 dL/gm, polydispersion 1.64), donated by Sun Pharmaceutical Advanced Research Center (SPARC), Baroda, India. Chloramphenicol was donated by Dey's Medical Stores (Mfg) Ltd. (Kolkata, India). Polyvinyl alcohol (PVA) and ethyl acetate were supplied by S.D. Fine Chemicals Ltd. (Mumbai, India) and Spectrochem (Mumbai, India) respectively. All other chemicals and solvents used were of analytical grade.

3.2. Preparation of nanoparticles

CHL-PLGA-NPs were prepared by an emulsion-solvent evaporation method (single emulsion technique) based on the classical procedure patented by Vanderhoff et al., 1979. Briefly, CHL was dissolved in 2 mL of ethyl acetate (organic solvent) containing PLGA, drug to polymer ratio (w/w) were kept at three levels (1 : 10, 2 : 10 and 3 : 10). The organic phase solution was slowly poured into 10 ml of aqueous 0.75% (w/v) PVA solution with slow magnetic stirring and sonicated using a pulse sonicator fitted with a round 3 mm diameter probe (model Labsonic M, B. Braun Biotech International; Melsungen, Germany) at 70 W amplitude intensity (frequency 30 kHz) and 0.7 s pulse cycle for 90 s on ice bath to produce the oil-in-water emulsion. The counter-diffusion of water into the drug plus polymer solution and counter-diffusion of ethyl acetate into the aqueous dispersion medium caused drug-loaded NPs to precipitate rapidly. A following slow stirring at room temperature for 4 h ensured the complete evaporation of organic solvent. The resulting colloidal dispersion of CHL-PLGA-NPs was centrifuged at 21,000 rpm (10 °C) to remove free drug and PVA. After careful decantation of supernatant, the residue was freeze-dried for 24 h using 2% sucrose as cryoprotectant. The dried CHL-PLGA-NPs were suspended in double distilled water and stored at 4 °C for stability study. Blank (without CHL) NPs were also prepared using same technique.

3.3. Preparation of free drug suspension (FDS)

FDS was prepared by dispersing 2%w/v of CHL in 10 ml of 0.75% w/v aqueous solution of PVA by mechanical stirring. This formulation was equivalent in drug content to CHL-PLGA-NPs containing 3 : 10 weight ratio of drug-to-polymer. Prepared FDS was analyzed for in vitro dissolution study using dialysis (described in 3.8).

3.4. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (JSM-5200, Tokyo Japan) was used to analyze particle size and surface topography as described by Voltan et al. 2007). The instrument was operated at 15 kV acceleration voltage. A concentrated aqueous suspension was spread over a slab and dried under vacuum. The sample was shadowed in a cathodic evaporator with a gold layer 20 nm thick. Photographs were elaborated by an image processing program and individual NP diameters were measured to obtain mean particle size.

3.5. Determination of Zeta-potential

Zeta potential of CHL-PLGA NPs and blank (without CHL) NPs was measured at 25 °C in an electrophoresis cell, which was fitted with two electrodes. A suitable amount of suspension of CHL-PLGA-NPs (50–100 µl) was diluted with 25 ml of filtered water and injected into the electrophoretic cell of the instrument (Zeta meter 3.0 plus inc, USA), where a potential of ±150 V was set. The Zeta-potential value was calculated by the instrument software. The procedure was repeated for blank NPs.

3.6. Determination of CHL entrapment efficiency

Aliquots of the freshly prepared suspension of CHL-PLGA-NPs (2 ml) were centrifuged at 21,000 × g for 25 min at 10 °C. The supernatant was anlyzed by an UV spectrophotometer (Genesis 10 UV, Thermoelectron Corporation, USA), set at 278 nm against a suspension of blank NPs. The amount of drug in supernatant represents the amount of drug not entrapped into NPs. By subtracting, entrapment efficiency was calculated. The experiment was performed triplicate for each batch and average was calculated.

3.7. Fourier transform infrared spectroscopy (FTIR) study

Fourier transform infrared (JASCO-FTIR, Model-8300) analysis was conducted to verify the possibility of interaction of chemical bonds between drug and polymer. Samples were scanned in the IR range from 400–4000 cm^{-1} and carbon black reference. The detector was purged carefully by clean dry helium gas to increase the signal level and reduce moisture.

3.8. In vitro release study

The diffusion cell model adapted to the spectrophotometer cubet, with 1 cm of optic way and 1 ml of volume, was used for the *in vitro* release of drug from CHL-PLGA-NPs. A cellulose acetate membrane (dialysis membrane with molecular weight cut off value of 5,000–10,000, Himedia-60) was adapted to the terminal portion of the glass cylinder of Franz Diffusion Cell by a rubber ring. Five ml of suspension of freeze dried CHL-PLGA-NPs in distilled water, sufficient for establishing sink conditions to the assay were taken into the donor compartment of the diffusion cell. The cylinder was coupled to the diffusion cell containing the receptor phase (0.2 M phosphate buffer solution pH 7.4) at 37 °C. At different time intervals, aliquots of 2 ml were withdrawn and immediately restored with the same volume of fresh phosphate buffer kept at 37 °C. The amount of CHL released was assessed by UV spectrophotometer (Genesis 10 UV, Thermoelectron Corporation, USA) set at 278 nm versus a calibration curve in the same buffer. Dissolution was carried out in triplicate for all three CHL-PLGA-NPs formulations and FDS.

3.9. Kinetics of drug release

To analyze the drug release mechanism, *in vitro* release data were fitted into zero-order, first order (Wagner 1969; Gibaldi et al. 1967), (Higuchi 1961; 1963) release equations and kinetic models.

3.10. Stability study

Suspensions of CHL-PLGA-NPs and blank NPs in double distilled water were stored at 4 °C for 3 months. After storage for 3 months, 3 ml of sample were taken to determine particle size, zeta potential value as described earlier. Additionally, CHL-PLGA-NPs formulations were subjected to drug leakage study. Suspension of CHL-PLGA-NPs (3 ml) was taken and centrifuged at 21,000 rpm for 25 min. The supernatant was assayed for drug

content in order to determine the leakage of entrapped drug molecules from NPs over time.

3.11. Statistical analysis

Experimental results were expressed as mean ± SD. Student's t-test was applied to determine the level of significance. One-way analysis of variance was also applied to check significant difference in formulations. Differences were considered statistically significant when $p < 0.05$.

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