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Nanoformulation and antibiotic releasing property of cefotaxime nanoparticles

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The objective of this study was to design nano-antibiotic to enhance their release from biomaterial agents. Cefotaxime was used as a model antibiotic substance in this carrier system. These nanoparticles were preformulated using different concentrations of polycaprolactone (PCL) and poly (vinyl alcohol) as coating material and prepared using double emulsion solvent evaporation method. The physiochemical properties of cefotaxime nano-antibiotic (Cefo-NPs) stability were determined. Results showed that the encapsulation efficiency of nanoparticles increased with increase in polymer concentration. In addition, dynamic light scattering (DLS) and atomic force microscope (AFM) indicated that the particles size were in the range of 189 to 219 nm. The drug release profile of Cefo-NPs shows rapidly the release behaviour under acidic environment. And thus make it a promising tool for control bacterial infection.

Key words: Polycaprolactone, poly (vinyl alcohol), cefotaxime, nanoparticle.

INTRODUCTION

Cefotaxime is a water soluble semisynthetic third generation of cephalosporin antibiotics, used for serious infections caused by susceptible strains of micro-organisms in lower respiratory infections, genitourinary infections, gynaecologic infections, skin infections, and central nervous system infections (Morelli, 2003). It acts by inhibiting bacterial cell wall synthesis.

Recent studies focused on using composite nanomaterials of silver-chitosan, chitosan-arginine, zinc-iron oxide, and polymer-antibiotics to control bacterial colonization and infection and potentially could be used as future therapeutic agents (Huang et al., 2011a, 2011b; Lellouche et al., 2012). Although, their mechanism of action is still unclear, but each of these materials has unique features that influence certain types of bacteria (Sivaraman and Ramamurthi, 2013). Additionally, different approaches were used to combine antibacterial materials to achieve desirable effects.

Nanocarriers, such as pH-sensitive nanoparticles, may represent one of these approaches that constitute an alternative strategy to overcome the difficulties that are related to microbial resistance and poor oral bioavailability (Ankola et al., 2010). It was found out that sustained release formulations prolong the action of the drug for a long period of time and also decrease the frequency of drug dosage. A sustained release formulation reduces the frequent drug administration (Shekhar et al., 2010).

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Table 1. Formulation plan or cefotaxime nanoparticles.

Nanoformulation	Cefotaxime (mg/ml)	PCL polymer (w/v %)	PVA (w/v %)
Cef-NP-1	5	1	0.2
Cef-NP-2	5	2	0.2
Cef-NP-3	5	1	0.3
Cef-NP-4	5	2	0.3

Poly ε-caprolactone (PCL) is a synthetic, biodegradable, biocompatible polymer often used in the formulation of nanoparticles. Nanoparticles prepared using PCL as a matrix was found to be larger than that prepared with other polymers. The colloidal suspension exhibited sustained release profile (Kumari et al., 2010). Since it is a low cost material, it is approved by the US Food and Drug Administration, and experience's slow degradation in the body (Mora-Huertas et al., 2010; Haddadi et al., 2008). The aim of this study was to prepare and characterize new nanoformulation of cefotaxime with PCL using solvent evaporation method.

MATERIALS AND METHODS

Preparation of standard curve of cefotaxime

From the stock standard solution, aliquots of 2, 4, 6, 8, and 10 ml were pipette out and the volume was made upto 10 ml with phosphate buffered saline (PBS) pH 7.4 to obtain concentrations in the range of 20 to 100 µg/ml. The absorbance of these solutions was measured at 256 nm by UV-Visible spectrophotometer, using phosphate buffer of pH 7.4 as blank. The absorbance values were plotted against concentration to obtain the standard graph.

Formulation of cefotaxime nanoparticles (Cefo-NPs)

Cefotaxime loaded nanoparticles were prepared with water in oil in water (w/o/w) double emulsion- solvent evaporation method. In this method, cefotaxime equivalent to 5% w/v was dissolved in 500 µl of PBS (0.01 M, pH 7.4) to form cefotaxime aqueous solution. The cefotaxime aqueous solution was emulsified in an organic phase consisting of 1 to 2% of the PCL polymer inorganic solvent di-cholo methane (DCM) to form primary water in oil emulsion. The emulsion was further emulsified in an aqueous 12.5 ml of poly-vinyl alcohol (PVA) stock solution (100 ml 2 to 3% w/v), as shown in Table 1, to form w/o/w emulsion. The emulsification was carried out using a micro tip probe sonicator (VC 505, Vibracell Sonic, Newton, MA, USA) set as 55 W of energy output for 5 min over an ice bath by adding the primary emulsion in dropwise to the 20 ml of phosphate buffer (0.01 M, pH 7.4). The emulsion was stirred for 18 h on a magnetic stir plate at room temperature to allow the evaporation of the organic solvent. Further 1 h vacuum drying was also performed to remove any residual organic solvent present. Any excess amount of PVA was removed by ultracentrifugation at 16000 rpm at 40°C for 20 min (Remi, India) followed by washing with double distilled water. The supernatant was collected and kept for an estimation of the amount of the drug which was not encapsulated. The recovered nanoparticulate suspension was lyophilized for two days (-800°C and <10 mm mercury pressure, LYPHILOCK 12, Labconco, Kansas City, MO, USA) to provide the lyophilized powder for further use (Niwa et al., 1994).

Percentage yield

The percentage practical yield was calculated to know about the percent yield or efficiency of any method, which would help in the selection of appropriate method of production. Practical yield was calculated as the weight of the dry nanoparticles recovered from each batch in relation to the sum of the starting material (Ramteke et al., 2006).

Percentage yield (PY) = (Practical yield/Theoretical yield) × 100

Fourier transform infra-red spectroscopy study

The Fourier transform infrared (Shimadzu-FTIR, Mo-del-8000 provided by Chemical Department, College of Science, Al-Nahrain University, Baghdad, Iraq) analysis was conducted to verify the possibility of chemical bonds between drug and polymer. Samples of pure cefotaxime, pure PCL and Cefotaxime-PCL physical mixture 1:1 were scanned in the IR range from 400 to 4000 cm^{-1} with carbon black as reference. The detector was purged carefully by clean dry helium gas to increase the signal level and reduce moisture.

Encapsulation efficiency (EE %)

The encapsulation efficiency, (EE %), was measured at wavelength of 256 nm by UV spectrometer (Spectronic Genesys 10 Bio, Thermo Electron Cooperation, WI, USA). The standard curve was prepared using drug concentration ranging from 1 to 10 mcg/ml and had a regression equation of $y = 0.076x$ with $R^2 = 0.988$. In each sample, EE% was measured by separating the aqueous phase with the colloidal one after centrifuge at 14,000 rpm for 30 min (VWR micro 18R, VWR Inc., West Chester, USA). The encapsulation efficiency of the drug loading was calculated using the following equation (Shabouri, 2003):

Encapsulation efficiency (%) = $(A^T - A^F) / A^T \times 100$

where A^T is the total drug amount and A^F is the nano encapsulated drug amount.

Particle size analysis

The size distributions along the volume mean diameters of the suspending particles were measured by dynamic scattering particle size analyser (Nanotrac Particle Analyzer 150, Microtrac Inc., PA, USA) (Alexis et al., 2008).

Determination of drug release profile of Cefo-NPs

One millilitre of the aforementioned Cef-PCL nanoparticles was centrifuged for 45 min at 20,000 rpm. After that, the precipitated

Table 2. Practical yield of nanoparticles.

Formulation	Practical yield (mg)	Total amount of ingredients (mg)	Percentage yield (%)
Cef-NP1	54	84	64.2
Cef-NP2	48	64	75
Cef-NP3	45	57	78.9
Cef-NP4	54	98	56.1

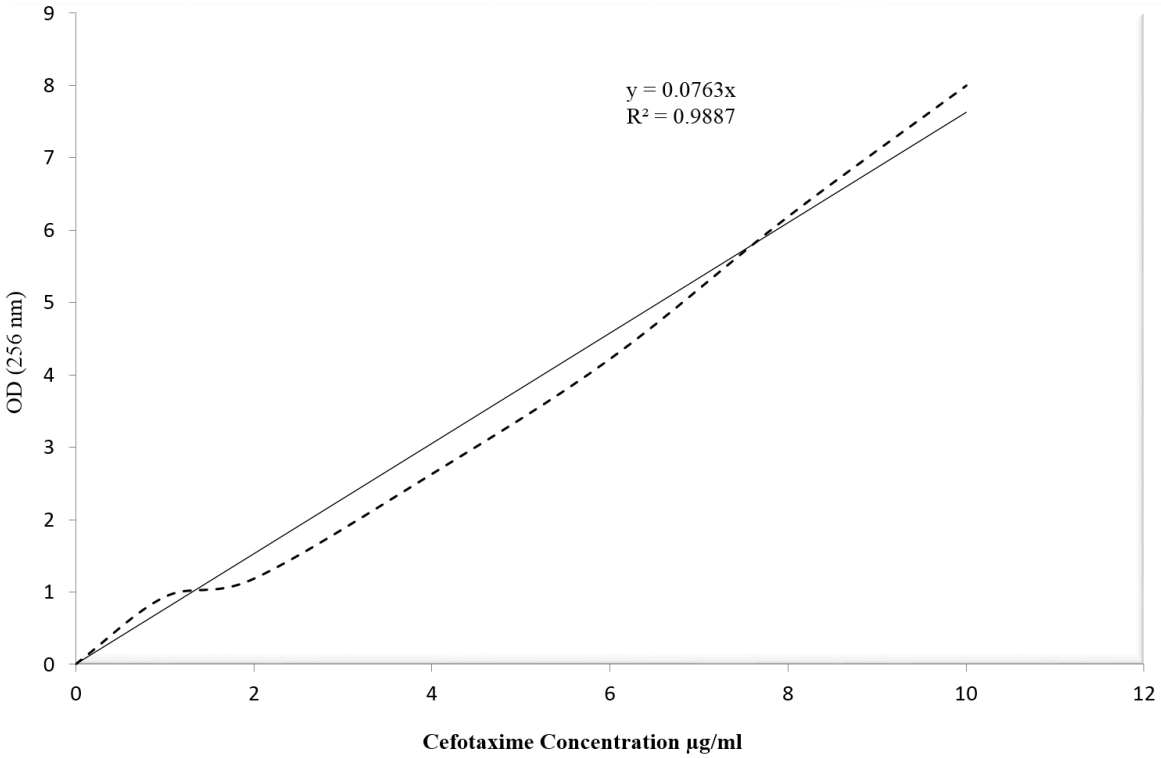

Figure 1. Standard calibration curve of Cefotaxime in phosphate buffer saline (7.4) at 256 nm.

Cef-PCL was redispersed in 1 ml of buffer solutions at pH 1.5, 4.5, 6.8, and 7.2. The dispersed particles were incubated for 1, 2, 4, 6, 12, 24, 48, 72 and 96 h at 37°C, which was conducted in triplicate. The amount of released drug was measured at 256 nm by UV-spectrometry. These results were shown as average ± standard deviation (n= 3). In addition, the drug loading efficiency (7.2 wt.%) was measured in the same manner. Briefly, cefotaxime weight was measured after lyophilisation and then dissolved in 1 ml of distilled water. The loaded amount of drug was measured by UV-spectrometry, using the following formula (Alexis et al., 2008):

Drug loading efficiency = (Weight of drug in nanoparticles/Weight of PCL-PVA) × 100.

RESULTS AND DISCUSSION

The w/o/w multiple emulsion solvent evaporation method is the mostly used technique for encapsulation of water-soluble drug. And it was the method of choice for the water-soluble cefotaxime drug (Kim et al., 2002). Four formulations of Cefo-NPs were formulated using different polymer emulsifier ratios, as shown in Table 2. The formulations are subjected to evaluation parameters like particle size, surface topography, drug encapsulation, and zeta potential.

Physicochemical characteristics, such as particle size and surface properties, all represented important parameters for determining the *in vitro* drug release, cellular uptake, and cytotoxicity of nanoparticles. The *in vivo* pharmacokinetics and biodistribution, influenced the therapeutic efficacy of the encapsulated drug (Youan et al., 2001). The absorbance measurement of cefotaxime standard solution containing 20 to 100 mcg/ml of drug in pH 7.4 PBS as shown in Figure 1 presented the standard calibration curve. The curve was found to be linear in the range of 20 to 100 mcg/ml at λ_{max} 256 nm. The regression coefficient value was found to be 0.9887.

Table 3. Encapsulation efficiency of cefotaxime.

Sample No.	Absorbance of drug loaded (O.D.)	Absorbance of free drug (O.D.)	Encapsulation efficiency (%)	Mean (±)	*STDEV	**STDREV
Cef-NP1	3.097	3	74.57	3.0485	0.068589	0.0485
Cef-NP2	3.019	2.987	90.77	3.003	0.022627	0.016
Cef-NP3	3.126	2.969	58.84	3.0475	0.111016	0.0785
Cef-NP4	3.098	2.965	61.65	3.0315	0.094045	0.0665

*STDEV: Standard deviation; **STDER: standard error.

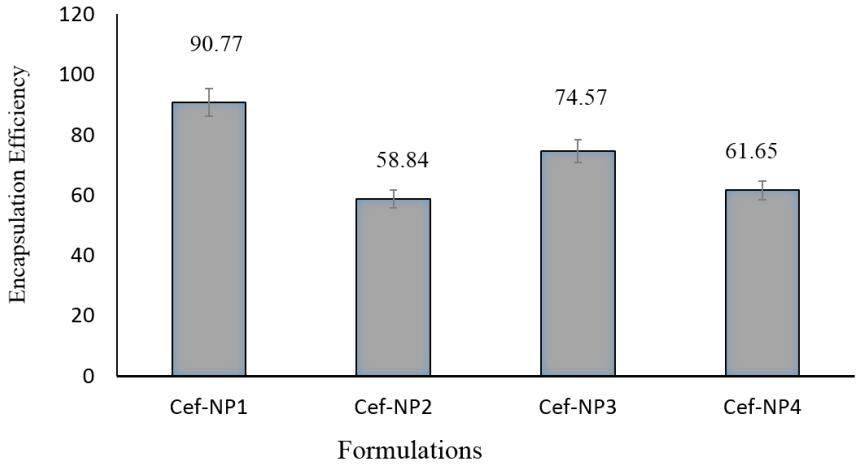

Figure 2. Drug encapsulation efficiency of nanoparticles.

Optimization of polymer and emulsifier ratio

For enhanced drug encapsulation, the results of this study showed that the drug/polymer and surfactant ratios and the concentration values influenced the final properties of the prepared nanoparticles. For PVA-emulsified PCL nanoparticle formation, drug concentration was kept constant at 5 mg per formulation, and PCL concentration varied from 1 to 2 g to give a drug/polymer ratio of 1:10 and 1:20. And for PVA concentration, it was from 1:1 and 1:2. These two different ratios of PCL and poly vinyl alcohol were applied. In this way, four formulations were prepared. The percentage of nano-particles yields varied from 55 to 54 mg corresponding to the amount of total amount of ingredients from 57 to 98 mg shown in Table 2. The results showed that the percentage of practical yield increased as the amount of polymer added to each formulation increased, although it may not be dependent upon PVA concentration in the formulation. Maximum yield was found to be 75 % for Cef-NP2 and 78.9% for Cef-NP3.

Drug encapsulation efficiency

The drug content in four batches of Cefo-NPs was studied. Table 3 and Figure 2 show the results of the drug encapsulation efficiency in each of these formulations. It was observed that the encapsulation efficiency increases with the increase in concentration of polymer in the formulations. The maximum encapsulation was 90.77 and 74.57% found in Cef-NP2 and Cef-NP1, respectively. Furthermore, the encapsulation efficiency increases as PVA concentration also increases on increasing the concentration of internal phase as indicated in Table 3 and drug content in Figure 2.

In this study, the result shows that encapsulation efficiency of the drug was depended on the solubility of the drug in the solvent and continuous phase. A similar observation was reported by Pignatello et al. (1997). The reason could be due to increase in size to encapsulate more drug and more surfactant similarly speed up the encapsulation process by enhancing the binding contact between drug and polymer in emulsion stage.

They are two important factors effect on the encapsulation efficiency, the type of polymer with the concentration and organic solvent selection. Large size nanoparticles are produced whenever high concentration of polymer in organic phase is applied (Dey et al., 2009). An interesting study found out that the stability of the emulsion droplets has an effect on the size of the formulated nanoparticles which is affected by miscibility of organic phase with water (Lourenço and Pinto, 2009). In this study, using dichloromethane as a water immiscible solvent led to the formation of nanoparticles by a true

Table 4. Size distribution of nanoparticles formulation with zeta potential.

Nanoformulation	Particle size (nm)	Zeta potential (mV)
Cefo-NP1	204	-45 (±) 0.78
Cefo-NP2	219	-58 (±) 1.2
Cefo-NP3	209	-34 (±) 3.1
Cefo-NP4	189.8	- 38 (±) 0.96

emulsification mechanism in which the larger emulsion droplets are broken into smaller droplets by the application of external energy.

Particle size and Zeta potential

The particle size of all formulated nanoparticles was estimated and was found to be in the range of 180 to 220 nm which indicates that they are stable. As the concentration of polymer increased, the particle size also increased. Furthermore, when the concentration of stabilizer (PVA) was increased, there was a decrease in the particle size from 219 to 189 nm. The polymer concentration in the internal phase is a crucial factor in increasing the size of nanoparticles. It was found out that high viscosity of the dispersion leads to higher resistance shear forces of emulsification. Therefore, at high viscous dispersions, the globule size of emulsions obtained will be higher, resultantly; after evaporation of solvent, higher size of nanoparticles were obtained. Earlier studies found out that particle size was proportional to the viscosity of the dispersed phase (Kim et al., 2002; Si and Samulski, 2008).

The stability study of the nanoformulation was performed by measuring the zeta potential of the nanoparticles using the zeta meter ± 3M. The results are evaluated in Table 4.

The significance value of using zeta potential is that its value can be related to the stability of colloidal dispersion. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, that is, the solution or dispersion will resist aggregation (Anacona and Silva, 2005).

Fourier transform infrared spectroscopy (FTIR)

Preformulation studies were carried out to study the compatibility of cefotaxime with PCL prior to the preparation of cefotaxime loaded nanoparticles. Infrared spectra of pure cefotaxime and PCL and their combination were studied. The FTIR studies show that there was no interaction between the drug and the polymer used. As indicated in the Figure 4. FTIR spectra of cefotaxime and its complex are similar and the main frequencies are shown in Figures 3 and 4. The lactam (C=O) band appears at 1780 cm^{-1} in the spectrum of cefotaxime, while the overlapped amide and ester (C=O) bands appear at 1640 cm^{-1}; the complexes show these bands at around 1720 to 1740 and 1630 to 1650 cm^{-1} ranges, respectively. All this suggests that coordination of the ligand occurs through the oxygen atom from the lactam carbonyl group rather than the amide and ester carbonyl groups. The lactam carbonyl bands were substantially shifted toward lower frequencies (40 to 60 cm^{-1}) relative to the value of the uncomplexed cefotaxime, while in the overlapped amide and ester carbonyl bands, the shifting was not significant (Chernysheva et al., 2003).

Atomic force microscopy (AFM) of Cefo-NPs

The AFM images showed that the nanoparticles formed as a result of solvent evaporation were spherical shaped and smooth in surface morphology, with an average diameter of less than 200 nm (Figure 5).The topography of cefotaxime nanoparticles were observed using AFM and it was found that the average diameter for Cef-NP1 was 109.8 nm, while it was 90.7 nm for Cef-NP2 formulation. In addition, the AFM of Cef-NP3 and Cef-NP4 nanoparticles were 103.9 and 74 nm (cumulative data details not published), respectively. Results showed no significant adsorption at the organic/aqueous phase boundary when DCM was applied as organic solvent. And thus, resulting in higher interfacial tension, causing agglomeration to occur, and hence the bigger particle sizes (Chernysheva et al., 2003). All the nanoparticles prepared with DCM as solvents were not uniform.

Sample preparation plays an important factor in order to get useful AFM images (Song et al., 2006). Samples must be thin enough and must adhere well to the surface, otherwise, the scanning process will producing artefacts. More details are presented in Chicea et al. (2012). In order to prepare the sample, a drop of nanofluid was deposited on a freshly cleaved mica substrate and stretched with a blade to form a very thin layer. The thin layer was left for 3 h to evaporate. The sample was attached to the AFM plate. First, a large area (5 µm × 5 µm) surface scan was carried out. Since the resolution used in the first scan is not good enough to image nanoparticles on a surface, several scans were done to select a flat area on the surface where there appear to be singular nanoparticles rather than aggregates, which were present, as well. Finally, a bigger resolution scan (512×512 pixels) was achieved and the topography is as shown in Figure 5. The scanned area is 1.0 µm × 1.0, several aggregates nanoparticles located on the scanned area is noticed.

In vitro drug release of Cefo-NPs

In vitro release profile of formulations is as shown in Figure 6. All the selected formulations showed sustained

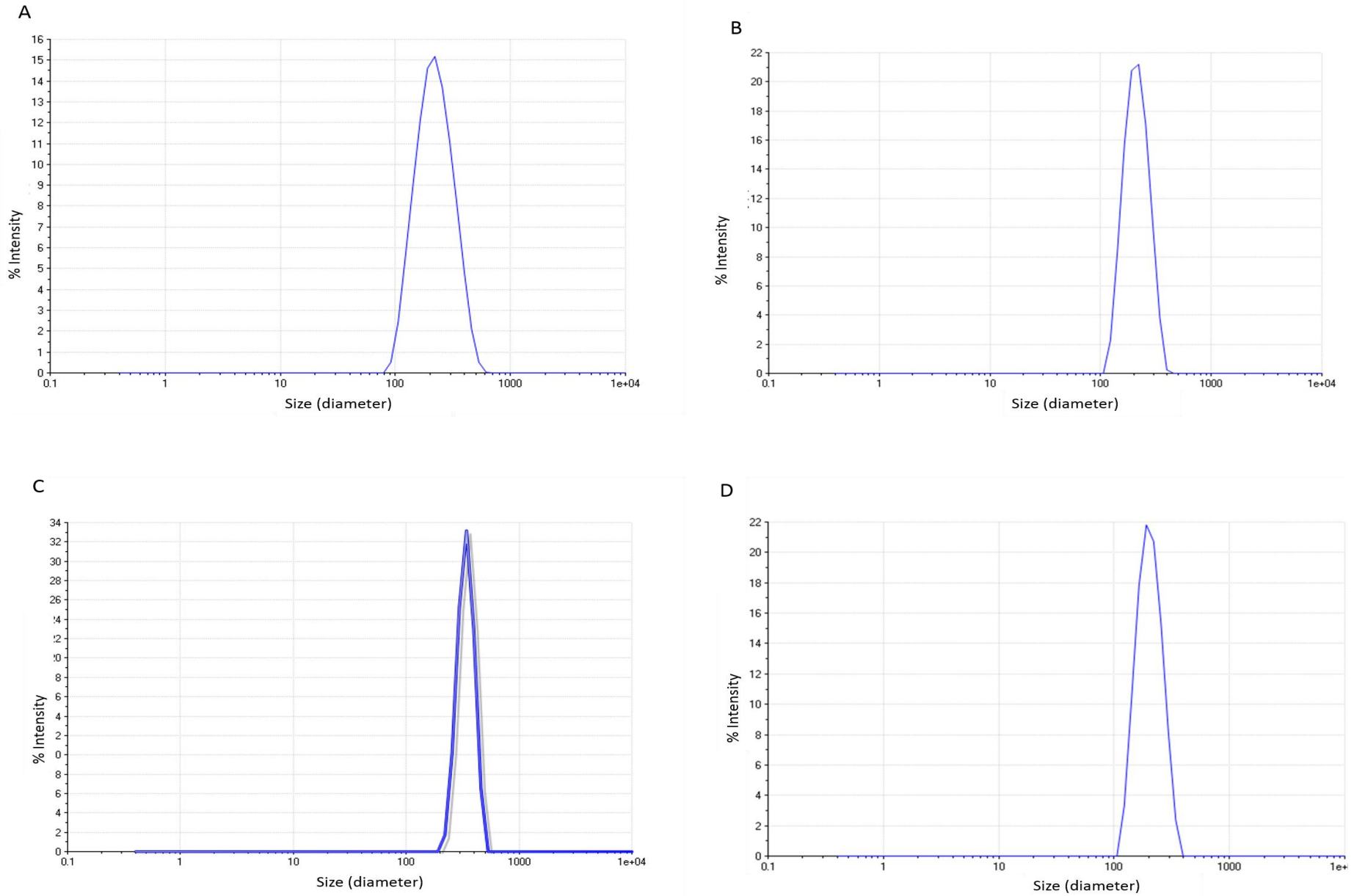

Figure 3. Zeta potential distribution curve of four formulated nanoparticles: (A) Cefo-NP1, (B) Cefo-NP2, (C) Cefo-NP3, and (D) Cefo-NP4.

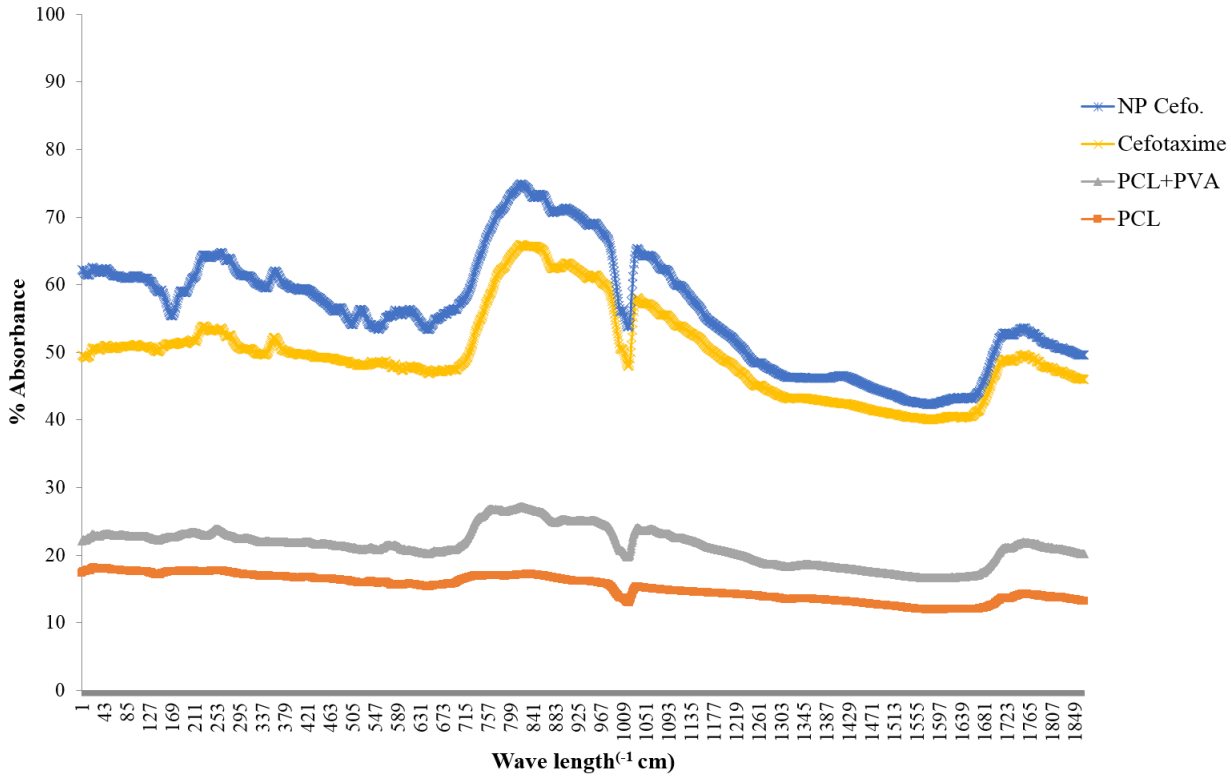

Figure 4. FTIR spectra of cefotaxime nanoparticle (NP-Cefo), Cefotaxime, Polycaprolactone-poly vinyl alcohol (PCL-PVA), and Polycaprolactone (PCL).

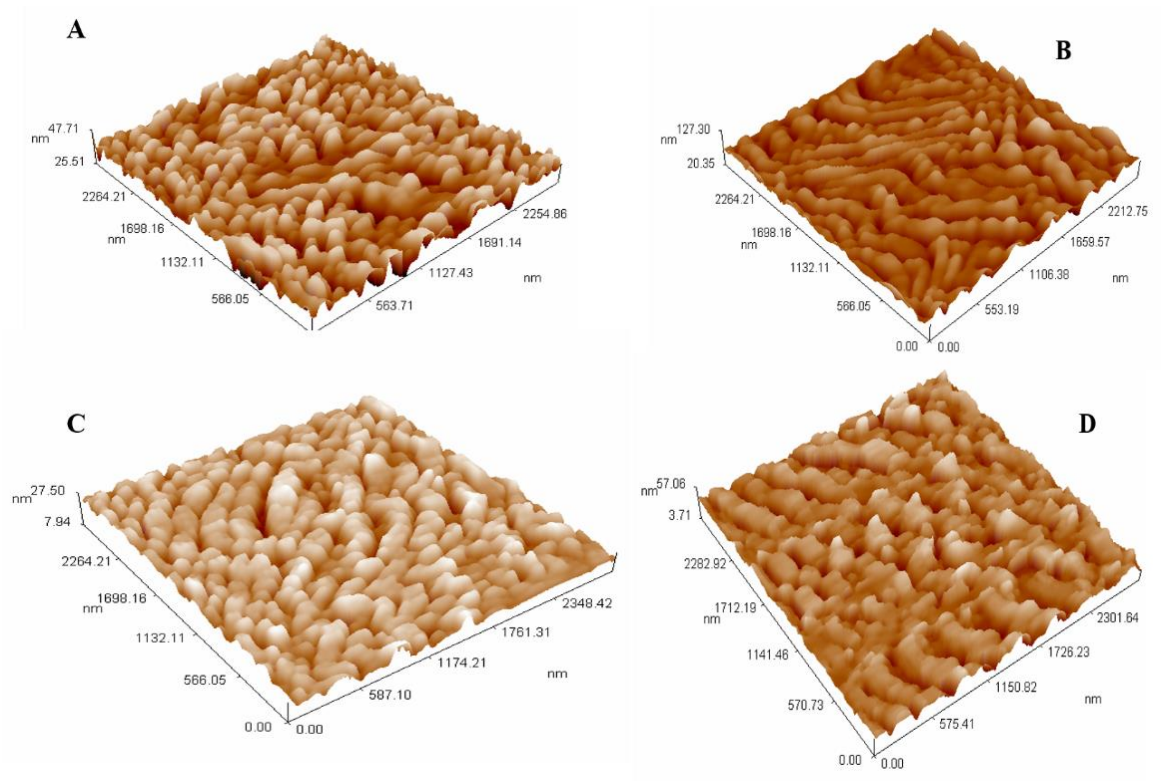

Figure 5. AFM plates for nanoformulation of cefotaxime: (A) Cefo-NP1, (B) Cefo-NP2, (C) Cefo-NP3, and (D) Cefo-NP4.

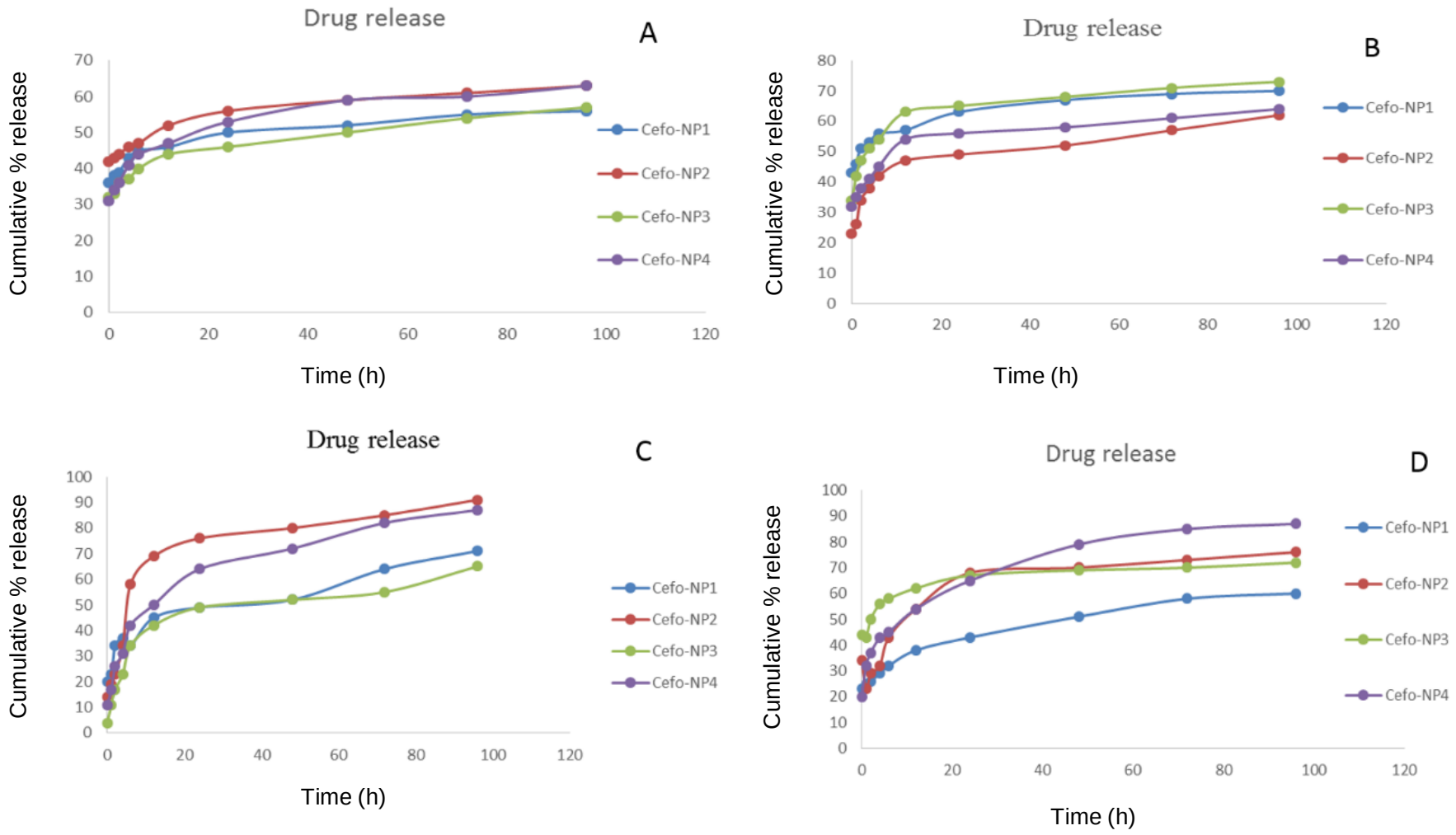

Figure 6. Cumulative % drug release vs. time for formulation of cefotaxime nanoparticles: A. pH (1.5), B. pH (4.5), C. pH (6.8), D. pH (7.4).

release patterns of drug in just 2 h. Except Cef-NP1 showed controlled release up to 8 h. Cef-NP4 formulation showed 95.37% release after 6 h. Whereas CCef-NP2 batch showed 94.29% release in 12 h. From the results of data fitting to various models, Cef-NP4 formulation shows controlled release. Cefotaxime was abruptly released from Cef-NPs under acidic conditions (pH 1.5) with about 60% of drug release within 6 h. Whereas only 30% was released at lower pH conditions (pH 1.5 and 4.5) during the same time period and both release profiles showed sustained

release patterns for 96 h.

pH-sensitive nanoparticles represented as un-conventional disperse systems for the nanometer size. Regarding to their effect in protecting the macromolecules from the acidity of stomach, clearance metabolism in the gastrointestinal tract reduce the drug side effect (Magenheim and Benita, 1991; Kreuter et al., 1989; McClean et al., 1998). Furthermore, due to their inherent pH sensitive property, the immerse drug will be able to release a specific pH within the gastrointestinal tract, next to its target window (Dai et al., 2004; Pereira et al., 2010). This system showed promising results to overcome many problems in relation to poor permeability of certain compounds and improve their oral bioavailability (Li et al., 2014).

In the present study, an attempt was made to develop Cefo-NPs for controlled release, improved drug efficacy, reducing drug toxicity and prolong the half lives in blood. And it was found out that these nanoparticles were successfully prepared by using oil/water solvent evaporation method. PCL is a suitable carrier for preparing nanoparticles of cefotaxime, as well as, solvent evaporation method has the advantages of being simple, fast and suitable for commercial scale up of nanoparticles. PVA seems to be the compatible stabilizer with clarithromycin. The presence of PVA stabilizes the emulsion droplets and prevents them for coalescing with other.

Conflict of Interests

The authors have not declared any conflict of interests.

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