ORIGINAL ARTICLE

Formulation, Optimization, and *In Vitro–In Vivo* Characterization of Furosemide-loaded Poly Lactic-co-glycolic Acid Nanoparticles for Sustained Delivery

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Abstract

Objective: In this study, the authors developed furosemide-loaded poly lactic-co-glycolic acid (PLGA) nanoparticles (NPs) for sustained delivery of furosemide. **Materials and Methods:** Furosemide-loaded PLGA-NPs were prepared by emulsion solvent evaporation method and characterized for particle size and size distribution, zeta potential, surface morphology, drug encapsulation efficiency, and drug release profile. *In vivo* study was performed in Charles Foster rats. **Results:** *In vitro* characterizations of the furosemide-loaded PLGA NPs showed that the mean particle sizes of the NPs ranged from 98.3 nm to 300.3 nm, the zeta potential values were in the range of −13.0–−27.1 mV, the encapsulation efficiencies were between 61.0 and 73.4%, and the drug release from the formulation was in the range of 40.3–80.7%. Scanning electron micrographs showed that the fabricated particles were spherical in shape. Urine output at the predetermined time showed a sustained effect of drug in PLGA NPs. **Conclusion:** The fabricated furosemide-loaded PLGA NPs were able to improve the sustained effect of the drug as indicated in *in vitro–in vivo* results.

Key words: Furosemide, nanomedicine, poly lactic co-glycolic acid, polymeric nanoparticles, sustained effect

INTRODUCTION

Nanoparticulate drug delivery systems have been investigated for the delivery of several therapeutic molecules for sustained release action.[1,2] Besides, till date, nanoparticles (NPs) have been extensively used for other applications such as drug delivery, tissue engineering, and imaging.[3] Their physiochemical properties, i.e. small size and large surface area are the criteria for these advances.[4] In drug delivery, they have been reported to significantly improve the sustained release property of drugs and minimize non-patient compliance, hence leading to more efficacious therapies.[5-8] Among the nanoparticulate drug delivery systems, polymeric NPs have been evidenced to be potential drug delivery systems for prolong release action.[9,10] Polymeric NPs are highly suitable due to their small diameter, spherical shape, and favorable zeta potential, prolonged drug release, and avoidance of reticuloendothelial system.[11-13] Polymeric NPs were also proved as safe and potential carriers for parenteral administration.[14]

Moreover, they also exhibit a good prospect for surface modification with different ligands that may provide improved pharmacokinetic parameters and are suitable to encapsulate and deliver a therapeutic agent.[15-17] Depending on the preparation process, polymeric NPs can be nanospheres or nanocapsules [Figure 1]. Nanospheres consist of matrix-like structure, in which active therapeutic ingredients can be adsorbed at their surface, entrapped, or dissolved in the matrix.[9,18] Nanocapsules consist of a polymeric shell and

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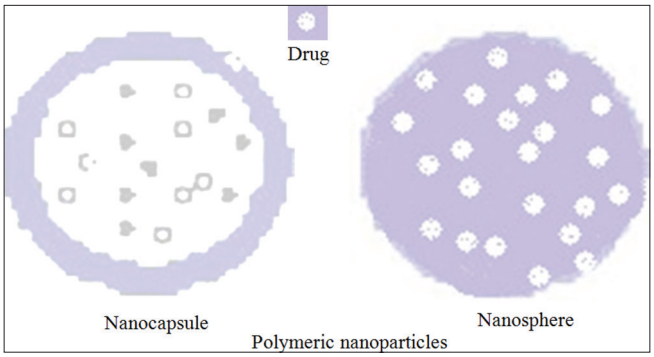

Figure 1: Types of polymeric nanoparticles

an inner liquid core. An active therapeutic ingredient is usually dissolved in the inner core or can also be adsorbed at the surface of the polymeric membrane.[18,19] The major advantage of utilizing NPs for drug delivery applications is their small size when taken up by cells, which may lead to significant drug accumulation at the target sites.[20] It has been evidenced that biodegradable polymers used for the fabrication of NPs lead to sustained drug release within the target site over a period of days or weeks.[21] The polymeric coating is supposed to lessen immunogenicity and could limit the phagocytosis of NPs by the reticuloendothelial system, leading to increased availability of blood drug levels in organs such as the brain, intestines, and kidneys.[4,22,23]

Furosemide is a potential loop diuretic utilized in the fluid management indicated in the edema associated with congestive heart failure, cirrhosis of the liver, renal disease, and hypertension.[24] Furosemide exhibits very short biological half-life[25] and therefore needed to administer repeatedly in disease like ascites where it may result serious side effects and non-patient compliance. Therefore there is need of formulations that can significantly sustain the release of furosemide.

It has been evidenced that poly lactic-co-glycolic acid (PLGA) has been recognized as the most attractive polymeric candidate utilized to fabricate devices for drug delivery, tissue engineering, and imaging applications.[26] PLGA is a Food And Drug Administration approved biocompatible and biodegradable polymer, possesses a broad range erosion times, and exhibits adjustable mechanical properties.[27] Particularly, PLGA has been broadly studied for the development of devices for sustained delivery of small molecule drugs, proteins, and other macromolecules. Recently, polymeric NPs as drug delivery vehicles have occupied the research interest due to their pharmaceutical advantages.[28] Polymeric NPs protect the degradation of therapeutic ingredient, ensuring stability of the drug, and hence improve the cellular availability of entrapped therapeutic ingredient for better efficacy. PLGA has been extensively explored commercially available polymer or synthesis of polymeric NPs.[29]

However, PLGA is considered most studied biodegradable polymer because of its long clinical experience, favorable degradation properties, and possible opportunity for sustained drug delivery.[30] Recent literature has shown that PLGA degradation has been utilized for sustained drug release.[26,30] Furthermore, the physical properties of the polymer-drug matrix can be refrained by directing the significant parameters such as polymer molecular weight, ratio of lactide to glycolide and drug concentration to attain a desired dosage, and release interval depending upon the drug type.[31,32]

Keeping the advantages of PLGA-based polymeric NPs, the concept has been explored for polymeric NPs of PLGA for sustained release action of furosemide in the present investigation.

MATERIALS AND METHODS

Materials

Furosemide was purchased from Sisco Research Laboratories, Mumbai, India. PLGA (D, L-lactide-co-glycolide) with a L/G ratio of 75:25 and Tween 80 were purchased from Sigma-Aldrich (Sigma-Aldrich, Saint-Louis, MO). Chitosan (extra-pure), 2-(N-morpholino) ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, acetic acid, and sodium hydroxide were obtained from Sisco Research Laboratories, Mumbai, India. All other chemicals used in this study were of analytical grade and used without further purification.

Methods

Preparation of furosemide-loaded PLGA particles

PLGA NPs were prepared by the conventional emulsion-solvent evaporation method [Figure 2]. Briefly, drug was dissolved in a polymeric solution of PLGA in ethyl acetate and emulsified in aqueous solution of Tween 80 for 1 or 2 min. The emulsion was stirred overnight on magnetic stirrer to evaporate the ethyl acetate to produce a suspension of NPs in the aqueous solution. The NPs were recovered by centrifugation at 12,000 rpm using Remi Centrifuge with a C-24 rotor for 20 min at 4°C, washed twice with MES buffer (100 mM, pH 5.5) and double-distilled water respectively. After each wash, the particles were recovered by centrifugation at 10,000 rpm for 20 min at 4°C. After the second wash, the NPs were obtained in MES buffer and mildly vortexed using a Spinix-vortex shaker to redisperse the pellets.[33]

NP characterization

Drug-excipient interaction analysis

The chemical interaction of furosemide with other excipients was assessed by Fourier -transform infrared (FTIR) spectroscopy. FTIR spectra of furosemide (pure) and lyophilized nanoformulations were obtained by conventional

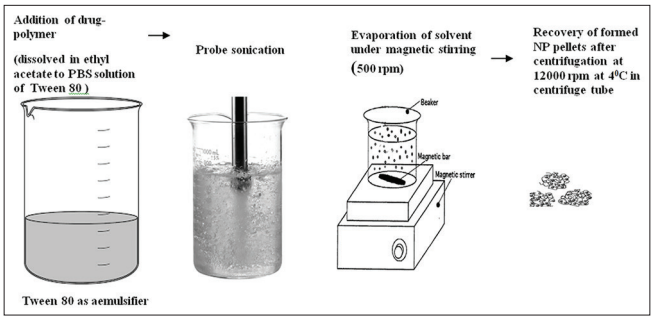

Figure 2: Schematic representation of emulsion solvent evaporation method to fabricate polymeric nanoparticles

KBr disk/pellet method (Shimadzu, Model 8400S, Tokyo, Japan). FTIR spectrum was obtained between the wavenumber of 400 and 4000 cm^{-1}.

Differential scanning calorimetry (DSC)

Furosemide, PLGA, and furosemide-loaded polymeric NPs prepared with surfactant tween 80 (vacuum evaporated sample) were subjected to DSC analysis to determine the physical state of the furosemide entrapped in the NPs. Samples were accurately weighed and sealed in aluminum pans. Thermograms were obtained by using a DSC Q 1000 from TA Instruments (USA). The thermal analysis was performed in a nitrogen atmosphere at a heating rate of 10°C/min over a temperature range of 50–250°C.

Particle size, polydispersity, and zeta potential

Particle sizes, polydispersity, and zeta potential of furosemide-loaded PLGA NPs were measured at 25°C by dynamic light scattering technique (Delsa Nano C-Beckmann Coulter). Data were fitted by the method of inverse "Laplace transformation" and CONTIN.

Scanning electron microscopy (SEM)

Surface morphology of obtained NPs was visualized using SEM under normal atmospheric conditions. The SEM (ZEISS Supra 40, Germany) was used at 20 kV. The slides were carbon coated and the images were obtained at 20KX magnification.

Transmission electron microscopy (TEM)

Surface morphology of furosemide-loaded PLGA NPs was performed by the TEM (JEOL USA).

Percentage encapsulation efficiency

Percentage encapsulation efficiency of the furosemide-loaded NPs was determined by vortexing (Spinix-Vortex) 10 mg of the obtained pellets dissolved in 25 ml of methanol for 10 min followed by centrifugation at a speed of 8000 rpm for 10 min to allow settling of the polymer as a precipitate. The supernatant containing drug was further diluted by methanol, and the absorbance was measured at 276 nm by ultraviolet (UV) spectrophotometer (Shimadzu, Japan). Drug loading and encapsulation of the NPs were calculated by the following formula.[34]

$$\% \text{ Entrapment efficiency } = \frac{\text{Practical drug content}}{\text{Theoretical drug content}} \times 100$$

In vitro drug release studies

The release patterns of furosemide from PLGA-NPs were performed by dialysis bag diffusion method. Briefly, 1 mL of NP suspension was taken into inner dialysis bag (cellulose membrane, molecular weight cut off 12–14 kDa, HiMedia Laboratories, Mumbai, India) and immersed into 20 ml of 0.1% (v/v) aqueous Tween 80 solution of alkaline phosphate buffer (pH 7.4). The entire system was kept on stirring of speed 100 rpm at 37°C. At predetermined time intervals, 5 mL samples were withdrawn from the outer compartment, and the same volume was adjusted with fresh phosphate buffer to maintain sink conditions. The furosemide content in the samples was determined using UV spectrophotometer, and the percentage drug release (%) was calculated at each time point interval.[35]

In vitro drug release kinetics

The mechanism of *in vitro* furosemide release from polymeric NPs in alkaline phosphate buffer (pH 7.4) can be determined by putting the dissolution data in kinetic models as:

Zero-order kinetics: $W = K_1 t$[36]
First-order kinetics: $\ln (100-W) = \ln 100 - K_2 t$[36]
Hixson–Crowell kinetics: $(100-W)^{1/3} = 100^{1/3} - K_3 t$[36]
Higuchi kinetics: $W = K_4 t^{1/2}$[37]
Korsmeyer–Peppas kinetics: $M^t/M^\infty = K_5 t^n$[38]
Irrespective of the involved mechanism, the rate of drug release can be given by a equation of:
$dw/dt = d/h$ (SCs)

Where dw/dt = release rate, W = drug released up to time t, D = drug molecule diffusion coefficient, S = effective surface area of drug with release medium, Cs = drug solubility in the medium, and h = diffusion path length.

For the NPs, the equation dw/dt=d/h (SCs) does not include all the release rate limiting factors such as rate of liquid penetration into the system, swelling, erosion, relaxation hydration, time and polymer dissolution.

In vivo study

In vivo study was approved and performed in accordance with the guideline of the animal ethics committee. All institutional and national guidelines for the Purpose of Control and Supervision on Experiments on Animals were followed (CPCSEA; Guidelines for Laboratory Animal

Facility) Registration No. Dean/2016/CAEC/1667. The rats were housed in metabolic cages, controlled conditions of temperature (25°C), and a 12:12 h light/dark cycle. The study was conducted in three groups consisting of four male Charles Foster rats (250–280 g). Animals were grouped as Group I - Rats administered with saline water (control), Group II - Rats administered with furosemide suspension, and Group III - Rats administered with optimized drug-loaded PLGA nanoformulation. Suspension of drug (6.6 mg/kg) and optimized nanoformulation (equivalent to 6.6 mg/kg of drug) was administered to animals by i.v. injection. Urine volume was recorded at pre-determined time intervals depicted in Figure 3.

RESULTS AND DISCUSSION

Particle size and surface morphology analysis

An increase in the polymer concentration leads to the increase in the particle size clearly evident in Table 1. Increase in particle size may be due to the increase in viscosity leading to increased emulsion droplet size ultimately leading to an increase in the particle size of the NPs.[39] High concentration of the polymer results in diminished shearing efficiency that may also be explained for increased size. The polydispersity index was in the range of 0.617 ± 0.044–0.782 ± 0.029 for different formulations obtained. Optimized stirring speed resulted in optimum particle-sized NPs (F-PLGA$_3$ 98 ± 11.4 nm) with high entrapment efficiency of 70.17 ± 1.37%, but at further higher speeds, drug leaching, i.e., low entrapment and particle agglomeration occurred that lead to increased particle size.

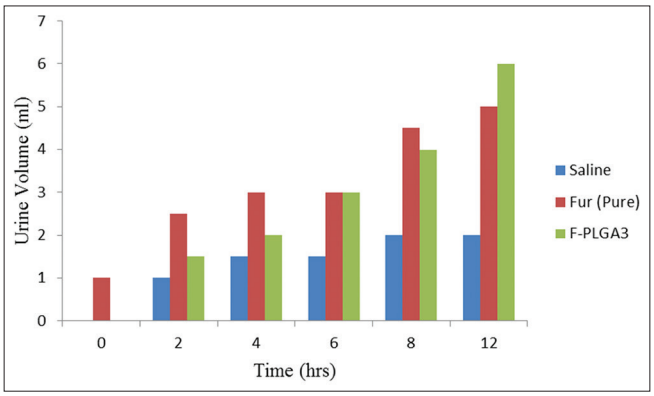

Figure 3: Time course of urine output in different groups of rats

The scanning electron microscopy images of the NPs are shown in Figure 4. The images depicted that the NPs were roughly spherical in shape. Images also display the smooth surface without any perceptible pinholes or cracks. It is also clear from the images that the higher amount of PLGA increased the particle sizes of NPs.

Figure 5 depicts the TEM image of furosemide-loaded PLGA NPs in 1 µm scale, which shows the fabricated NPs sizes <100 nm. The surface of the obtained NPs was smooth without the presence of any significant cracks.

Drug-excipient interaction studies

FTIR analysis

It has been reported that the native furosemide spectrum shows sharp characteristic peaks at 3400 cm^{-1}, 3260 cm^{-1}, 1665 cm^{-1}, and 1560 cm^{-1} [Figure 6a].[40,41] FTIR spectrum of PLGA is characterized by a main peak at 1752.07 cm^{-1} [Figure 6b] due to the presence of ester group.[42] The prominent peaks representing furosemide and PLGA appear in the spectra of furosemide-loaded PLGA NPs and did not show any significant shifting in the position of the absorption peaks [Figure 6c].

DSC analysis

Figure 7 shows DSC curves of furosemide, PLGA, and furosemide-loaded PLGA NPs. Furosemide showed a sharp endothermic peak at furosemide melting temperature of 206°C which confirms its crystalline structure [Figure 7a].[24]

Table 1: Formulae, particle size, zeta potential, PDI, and percentage encapsulation of the furosemide-loaded PLGA (F-PLGA) formulations

Formulation code	Drug: polymer ratio (mg)	Mean particle size* (nm)	Zeta potential* (mV)	Polydispersity* index	% encapsulation*
F-PLGA1	0.5:01	150.3±11.6	−27.1±2.3	0.7780±0.022	64.73±2.13
F-PLGA2	01:0.5	124.4±10.1	−22.3±1.4	0.7519±0.021	68.03±1.13
F-PLGA3	0.5:0.5	98.3±11.4	−13.0±1.0	0.6175±0.044	70.17±1.37
F-PLGA4	01:01	110.2±15.6	−25.1±1.7	0.7313±0.029	73.42±1.27
F-PLGA5	01:02	238.3±11.10	−23.3±1.1	0.7013±0.023	68.29±2.13
F-PLGA6	02:01	231.3±13.6	−15.5±1.0	0.6512±0.056	65.06±1.43
F-PLGA7	01:03	300.3±13.1	−25.1±1.0	0.7823±0.029	61.02±1.02
F-PLGA8	03:01	134.1±14.2	−21.3±1.5	0.7419±0.023	63.38±2.82

*Mean±SD, *n*=3, SD: Standard deviation. PDI: Polydispersity index, PLGA: Poly lactic-co-glycolic acid

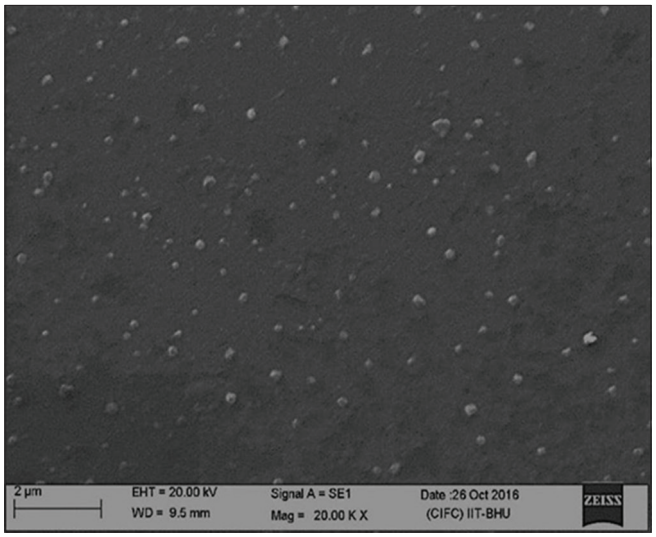

Figure 4: Scanning electron microscope image of furosemide-loaded poly lactic-co-glycolic acid nanoparticles

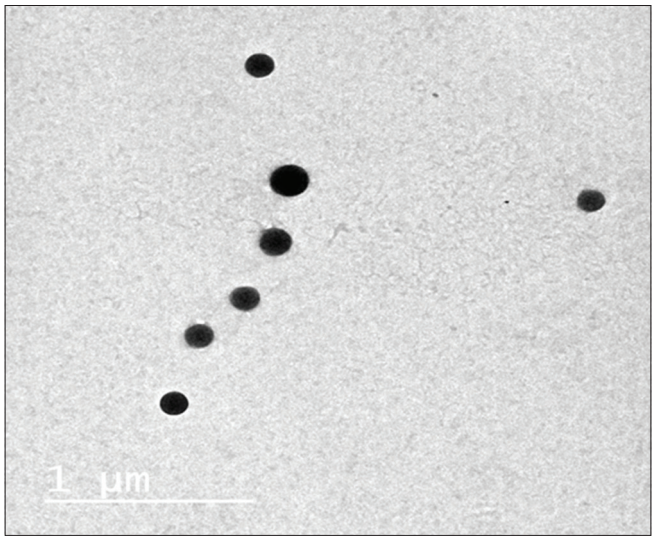

Figure 5: Transmission electron microscope image of furosemide-loaded poly lactic co-glycolic acid nanoparticles

The DSC curve of PLGA has shown the absence of any melting peak which confirmed an amorphous nature of the polymer [Figure 7b]. Furthermore, the thermograms of furosemide-loaded PLGA NPs [Figure 7c] did not show the melting peak of the furosemide.

Percentage encapsulation efficiency

Percentage encapsulation efficiency of the furosemide-loaded PLGA NP is summarized in Table 1 according to which it can be summed that the formulation F-PLGA$_4$ (1:1 ratio) showed 73% entrapment of the drug which was the best among all the prepared batches. However for the particle size (98.3 nm), batch F-PLGA$_3$ (0.5:0.5) is the best formulation. The reduced entrapment efficiency in other batches may be due to the saturation effect of the polymer or drug leaching.

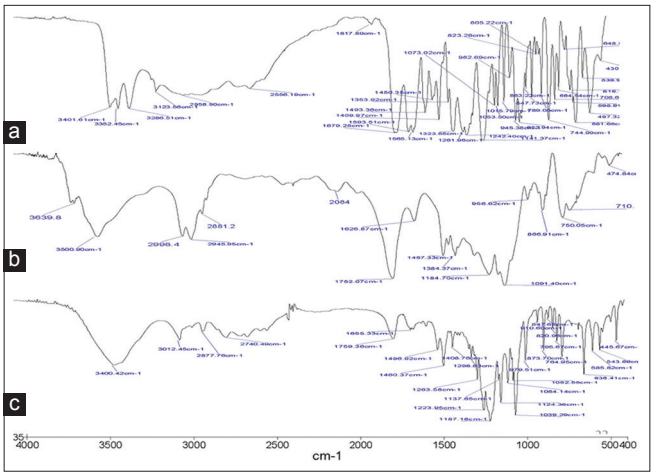

Figure 6: Fourier-transform infrared spectrum of furosemide (a), poly lactic-co-glycolic acid (PLGA) (b), Furosemide-loaded PLGA nanoparticles (c)

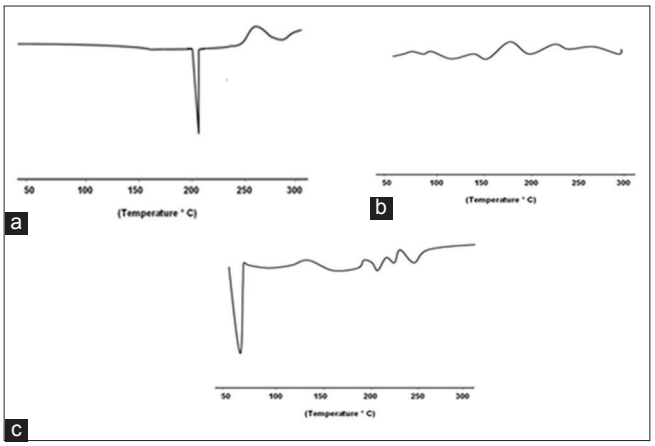

Figure 7: Differential scanning calorimetry thermograms: (a) Furosemide (pure), (b) poly lactic-co-glycolic acid (PLGA), (c) Furosemide-loaded PLGA nanoparticles

In vitro drug release

The drug release from the furosemide-loaded PLGA NPs is presented in Figure 8, which depicts the reduced drug release from the NPs when the polymer concentration is increased, which may be explained as the formation of rigid polymer matrix at higher polymer concentration which leads to low polymer dissolution, thus causing a slower drug release from the NPs. F-PLGA$_3$ exhibited a better drug release profile (80.76 ± 1.04%) at a drug:polymer ratio of 0.5:0.5.

On applying *in vitro* drug release data to various kinetic models to predict drug release mechanism and kinetics, the plots released amount versus square root of time was found linear determining diffusion controlled mechanism of drug release. Furthermore, the correlation coefficients (r^2) were found in the range of 0.979–0.986 for various formulations. Furthermore, first-order kinetics was seemed to be followed when straight lines were obtained ($r^2 > 0.94$) by plotting log

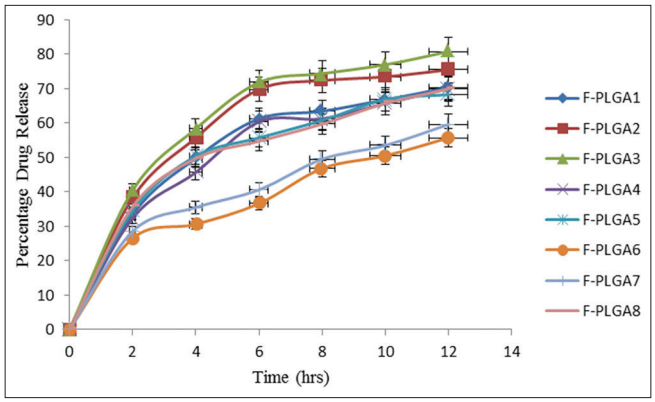

Figure 8: Percentage drug release profile of furosemide-loaded poly lactic-co-glycolic acid (PLGA) nanoparticles (batch F-PLGA$_1$- F-PLGA$_8$)

percentage of drug remaining to be released versus time.

CONCLUSION

From the study discussed above, it can be concluded that the formulated furosemide-loaded PLGA NPs can be utilized for the sustained delivery of the drug. PLGA-based NPs showed sustained release effect of the furosemide with an optimum particle size of 98.3 ± 11.4 nm for the selected batch F-PLGA$_3$. There was no drug-polymer interaction as revealed from the FTIR studies. DSC analysis showed that the furosemide was completely conformed in the polymer in the formulation and no crystalline form was present. Drug entrapment was 70% for a formulation which has 0.5:0.5 drug:polymer ratio of particle size 98.3 nm and was further selected for *in vivo* study. Urine output at different groups of rats showed the sustained effect of furosemide up to 12 h. Future perspective of the study indicates to assess the pharmacokinetic study by high-performance liquid chromatography and defensive value of furosemide-loaded PLGA NPs against systemic toxicity.

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