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In situ forming implant for controlled delivery of an anti-HIV fusion inhibitor

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ABSTRACT

An injectable, phase sensitive, *in situ* forming, implantable delivery system was developed for enfuvirtide, a therapeutic peptide used in the treatment of HIV infection. The development studies were carried out using poly (D,L-lactide-co-glycolide), a smart, biodegradable polymer. Different formulations were designed, prepared and evaluated by employing response surface, optimal design of experiment technique. The optimized formulation was identified and validated for its performance by using numerical optimization technique. The *in vitro* evaluation parameters included rheology, compatibility studies, drug release as well as conformational and physicochemical stability studies. *In vivo* pharmacokinetic parameters and biocompatibility studies were determined in rat models and were statistically analyzed. It was found that the optimized formulation extended the enfuvirtide release and maintained the drug plasma concentration within therapeutically effective range up to 48 h. The optimized formulation maintained physicochemical and conformational stability for at least 6 months and was biocompatible with the animal tissue.

1. Introduction

The objective of present study was design, development and evaluation of phase sensitive, *in situ* forming implants (ISFI) for the short-term, sustained, subcutaneous delivery of enfuvirtide. Enfuvirtide (T-20) is the first Food and Drugs Administration (FDA) approved member of the new antiretroviral drugs classified as entry inhibitors or fusion inhibitors (USFDA, 2010). It is a synthetic peptide with molecular weight 4492 Da and molecular formula $C_{204}H_{301}N_{51}O_{64}$. It is composed of a linear sequence of 36 naturally occurring L-amino acid residues with an acetylated N-terminus and a carboxamide C-terminus (EMEA, 2006). Enfuvirtide acts by inhibiting the fusion and entry of retrovirus through the cell membrane of target $CD4^+$ cells, thus controlling the multiplication of HIV and further advancement of the disease (Manfredi and Sabbatani, 2006). Enfuvirtide is administered, as 90 mg twice-a-day *s.c.* injection due to its short half-life of 3.5–4 h. Treatment of HIV positive patients requires long-term therapy with single or multiple antiviral agents. Consequently, the patient is required to take two or more injections per day that may lead to non-compliance by the patient. The local site inflammatory reaction caused by frequent injection of the drug may also lead to complete discontinuation of enfuvirtide therapy. Enfuvirtide causes significant inflammation and pain at the injection site, which normally subsides within a few days but in many cases the severity of the reaction, is high enough to force the physician to discontinue the therapy (Foy and Juethner, 2004).

One of the novel drug delivery systems that are successfully used for the effective and sustained delivery of peptides and proteins are subcutaneous (*s.c.*) or intramuscular (*i.m.*) solid depot implants. These systems could provide sustained release of macromolecules ranging from a few days to several months, thus avoiding the daily multiple injections. Furthermore, encapsulation of such labile molecules in the polymer matrix of the delivery systems provides protection against enzymatic degradation (Al-Tahami and Singh, 2007). However, these implants present with a major drawback concerning patient acceptability and compliance. Since, implants are solid, rigid structures, a minor surgery is required for their subcutaneous installation and for removal after the drug depletion if the implant is formed using a non-biodegradable material.

The biodegradable, smart polymer based, *in situ* forming, implantable, liquid depot systems for controlled, subcutaneous (*s.c.*) or intramuscular (*i.m.*) delivery can be a promising solution to the formulation challenge of the polypeptides and acceptability issue of non-biodegradable solid implants. Unlike non-biodegradable controlled delivery systems, surgical removal of drug-depleted biodegradable systems is not required as these systems spontaneously degrade into non-toxic byproducts in the body. Stimuli-sensitive polymers or "Smart Polymers" represent a rapidly growing area with enormous technological and commercial potentials in pharmaceutical and biomedical fields (Langer,

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1995). These materials are useful to create unique functional polymeric systems, mimicking the natural feedback systems, which can sense one or more stimuli, change their structures and functions in direct response to the stimuli. Their responses are often accompanied by dramatic changes in shape, surface characteristics, solubility, molecular self-assembly, and sol-to-gel transition (Langer and Peppas, 2003).

Among various systems developed by using stimuli sensitive polymers, phase sensitive, *in situ* forming systems offer various advantages such as ease of manufacture, ease of administration, less stressful manufacturing conditions for sensitive drug molecules, high loading capacity and prolonged delivery periods (Singh and Singh, 2004). This approach employs a water insoluble biodegradable polymer, such as poly(D,L-lactide)/poly(D,L-lactic acid) [PLA], poly(D,L-lactide-co-glycolide)/poly(D,L-lactic acid-co-glycolic acid) [PLGA] and poly(D,L-lactide-co-ε-caprolactone) [PLC], dissolved in a pharmaceutically acceptable solvent to which a drug is added, forming a solution or suspension. After injection of the formulation into the body, the water miscible organic solvent dissipates and water penetrates into the organic phase. This causes phase separation and precipitation of the polymer forming a depot at the site of injection (Eliaz and Kost, 2000; Ravivarapu et al., 2000).

It was thus, hypothesized that formulating an ISFI system for enfuvirtide would prolong its release over an extended period and would prevent excessive contact of the drug with the tissue by entrapping most of the drug in the biocompatible polymer matrix releasing small quantity of drug at a time and thus reducing the intensity of injection site reaction and pain.

2. Materials and methods

2.1. Materials

Enfuvirtide was purchased from Bioworld, bioPLUS Fine Research Chemicals, USA and PLGA50:50 [intrinsic viscosity 0.19 dL/gm; MW 6000 Da] was purchased from Lactel absorbable polymers, Durect Corporation, Cupertino, USA. Dimethyl sulfoxide (DMSO), benzyl alcohol (BA), benzyl benzoate (BZ) and triacetin (TR) were purchased from SD Fine Chemicals Ltd., Mumbai, India. Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were purchased from Qualigens Fine Chemicals Ltd., Mumbai, India. Arginine hydrochloride and *n*-nonyl-β-D-glucopyranoside were purchased from Sigma–Aldrich Chemicals Pvt. Ltd., Mumbai, India. All other chemicals were reagent grade. Membrane filters (0.22 μm, 0.45 μm, 1.2 μm, 30 μm) were purchased from Advanced Micro Devices, AmbalaCantt, India.

2.2. Methods

2.2.1. Solubility studies of enfuvirtide

The equilibrium solubility of enfuvirtide was determined in different organic and aqueous solvents and buffer solutions. The saturated solution with excess drug was shaken on a horizontal shaker at 37 °C for 48 h, centrifuged, diluted and analyzed for enfuvirtide concentration by spectrofluorimetric method. The results of solubility studies of enfuvirtide were used to determine the suitable solvent system for the preparation of ISFI formulations and suitable release media for *in vitro* studies.

2.2.2. Spectrofluorimetric method for enfuvirtide analysis

The spectrofluorimetric method for enfuvirtide analysis was carried out using a Spectrofluorimeter (F-2500, Hitachi, Japan) equipped with a xenon arc lamp, preloaded with data interpreting software (FL Solutions v 2.0). Relative fluorescence intensity (RFI) was used in all the spectrofluorimetric measurements of enfuvirtide. The values of excitation wavelength (λ_{exc}) and emission wavelength (λ_{emi}) were 280 nm and 350 nm, respectively (Salome Veiga et al., 2004a,b). The spectrofluorimetric method for enfuvirtide analysis was validated for linearity, accuracy and precision (Bende et al., 2007).

2.2.3. Micronization of enfuvirtide

In situ micronization by controlled association or non-solvent precipitation was selected for particle size reduction of enfuvirtide. According to this method, 100 mg enfuvirtide was dissolved in 1 mL dimethylformamide (DMF), an excellent solvent for enfuvirtide. This solution was then added to 5 mL triple distilled water, a non-solvent for the drug. The drug precipitates out on contact with the non-solvent due to its poor solubility in it (Moshashaee et al., 2000). The dispersion was ultracentrifuged at 10,000 rpm to separate the fine drug precipitate as sediment. The supernatant was discarded and the sediment was washed three times with chilled, triple distilled water followed by drying in vacuum on desiccators. The particle size of micronized drug was compared with that of non-micronized drug using an optical microscope and Malvern Mastersizer.

2.2.4. Preparation of enfuvirtide ISFI formulations by homogenization method

Since, other methods such as ultrasonication and use of unmicronized drug failed to produce the required formulation, only the successful method of formulation by homogenization is reported here. The overall procedure is described diagrammatically in the graphical abstract of this article.

Weighed amount of PLGA 50:50 (30%, w/w) was added to the DMSO taken in a screw-capped glass vial. The vial was incubated at 37 °C for 24 h to dissolve the polymer. After 24 h, micronized drug (50%, w/w based on polymer weight) was added to the polymer solution and homogenized for 15 min by using Ultra Turrax® at 10,000 rpm to dissolve any remaining polymer and disperse the drug. This formulation was named P4.

2.2.5. Formula optimization of enfuvirtide ISFI system by design of experiment (DoE) method

Based on the results of studies carried out to select suitable drug type and preparation method, different formulations were prepared to optimize drug: polymer ratio and solvent type. The quantity of drug was constant in all these formulations (15%, w/w based on total formulation weight). The experiments were designed by using an experimental design software (Design Expert®, v 8.0.1, Stat-Ease, USA). An I-Optimal Response Surface Design was employed to obtain 9 different factor combinations and 9 replicates where two independent variables were studied at three levels (Stat-Ease, 2010). The details of studied factors and levels are shown in Table 1. Different factor combinations that were obtained and experimentally run to measure the responses Y_1 (percent enfuvirtide release at 24 h), Y_2 (percent enfuvirtide release at 48 h) and Y_3 (viscosity) are given in Table 2.

2.2.6. In vitro enfuvirtide release from ISFI systems

Enfuvirtide ISFI systems equivalent to 360 mg enfuvirtide were injected into 100 mL pH 7.4 phosphate buffer containing 0.01% sodium azide, through a 21 gauge needle, within 20 s and shaken at 37 °C in an incubator shaker (Graves et al., 2007). Samples of 1 mL volume were withdrawn and replaced by fresh release media at 1, 2, 4, 8, 12, 18, 24, 32, 40 and 48 h. The enfuvirtide concentration in the samples was determined by spectrofluorimetric analysis at excitation wavelength of 280 nm and the emission wavelength of 350 nm. Amount of enfuvirtide in the released samples was obtained from

Table 1
Factors and levels used to design the experiment for the preparation of enfuvirtide ISFI formulations.

Factors	Factor type	Levels		
		L1	L2	L3
Polymer:Drug ratio (X_1) (wt. ratio)	Numeric	1:1	1.5:1	2:1
Solvent type (X_2)	Categoric	DMSO	DMTR 60:40	BABZ 70:30

the standard curve and was corrected for withdrawn samples using Eq. (1):

$$C_n = C_0 + \frac{Vs}{Vt}\left(\sum_{n-1}^{1} C_0\right) \quad (1)$$

where C_n is the corrected concentration; C_0 is the uncorrected concentration; V_s is the volume of sample withdrawn; and V_t is the total volume of dissolution medium (Singh and Singh, 1998; Singh et al., 1997).

2.2.7. *In vitro release kinetics of enfuvirtide ISFI formulations by mathematical modeling*

The *in vitro* drug release data obtained from ISFI systems were fitted to various release kinetics models (Higuchi, 1963; Hixson and Crowell, 1931; Korsemeyer et al., 1983; Peppas, 1985; Peppas and Sahlin, 1989), *viz*., first-order, Higuchi, Hixson–Crowell cube root, Korsemeyer–Peppas and zero-order mathematical models. Selection of a suitable release models for enfuvirtide ISFI systems was based on values of R^2 (correlation coefficient), k (release constant) and n (diffusion exponent) obtained from curve fitting of release data. The model fitting was accomplished using MS Excel 2007 and PCP Disso ver 3.0 software (Ketkar et al., 2003).

2.2.8. *Viscosity of enfuvirtide ISFI formulations*

The viscosity of enfuvirtide ISFI formulations (4 mL) was measured by using a rheometer (Rheolab QC, DG 26.7 geometry and Rheoplus/32, v 3.40 software). The shear rate was varied from 2 to 100 1/s for 3 min. Temperature was maintained at 25 ± 2 °C using a fluid bath surrounding the outer cylinder. Viscosity of the prepared formulations was calculated using Eq. (2):

$$\text{Viscosity} = \frac{\text{Shear stress}}{\text{Shear strain}} \quad (2)$$

2.2.9. *Statistical analysis*

The formulations prepared according to the design were analyzed by using Design Expert® ver 8.0.1 software package. The effect of formulation variables on the response variables was statistically evaluated by one way ANOVA at 0.05 levels (Daniel, 1983a; Stat-Ease, 2010). The design was evaluated by response surface method using following polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 \ldots \quad (3)$$

where Y is the response variable, β_0 the constant and β_1, β_2, β_3 are the regression coefficients. X_1 and X_2 stand for the main effect, X_1X_2 are the interaction terms and show how the response changes when two factors are simultaneously changed (Singh and Ahuja, 2004; Singh et al., 2005). The equation for each response parameter was generated using one-way ANOVA and multiple linear response analysis (MLRA) (Daniel, 1983b). The predictor equation containing only significant terms were generated by backward elimination procedure.

A numerical optimization procedure using desirability approach was used to locate the optimal settings of the formulation variables in order to obtain the desired response. Constraints for the drug release at 24 h and 48 h were set in the range of 45–60 and 75–100, respectively, and viscosity was set to minimize over the range.

2.2.10. *Validation of optimization results*

The formulation predicted to be optimal by optimization methodology was prepared and analyzed to validate the response. Predicted and experimental values of different response variables were compared and percent prediction error was calculated.

2.2.11. *Evaluation of optimized enfuvirtide ISFI formulation*

2.2.11.1. Rheological studies. Rheological investigations are basically concerned with determination of the relationship between shear stress, stress rate and viscosity (Plaizier-Vercammen et al., 1989). These parameters were determined using a rheometer where the shear rate was varied from 2 to 100 1/s for 3 min and the temperature was maintained at 25 ± 2 °C. Power law equation was used to describe non-Newtonian flow characteristics of gels (Martin et al., 1991; Wood, 1987). It is given as:

$$\gamma = K\tau^n \ldots \quad (4)$$

Taking logarithm on both the sides

$$\text{Log}\,\gamma = \text{Log}\,K + n\,\text{Log}\,\tau \ldots \quad (5)$$

where τ is shear stress, γ is shear rate, K is consistency index and n is flow index.

Consistency index and flow index were calculated by plotting graphs of log shear rate *vs* log shear stress where antilog of intercept and the slope of line were consistency index and flow index, respectively (Kapil et al., 2009). Flow index indicated the measure of deviation from Newtonian behavior ($n = 1$). Shear thinning (pseudoplastic) and shear thickening (dilatant) behavior were indicated by $n < 1$ and $n > 1$, respectively.

Table 2
Response variables Y_1, Y_2 and Y_3 obtained by evaluation of different enfuvirtide ISFI formulations prepared as per I-optimal response surface experimental design.

Batch no. ($n = 2$)	PLGA:Drug wt. ratio (X_1)	Solvent type (X_2)	Release 24 h (Y_1) %; ($n = 3$) ± S.D.	Release 48 h (Y_2) %; ($n = 3$) ± S.D.	Viscosity (Y_3) cP; ($n = 3$) ± S.D.
E1	1.5:1	BABZ	52.64 ± 2.16	78.25 ± 2.27	446 ± 1.39
E2	1:1	DMSO	68.36 ± 1.36	96.77 ± 1.65	352 ± 1.52
E3	2:1	DMSO	48.37 ± 1.43	72.57 ± 1.50	486 ± 0.94
E4	1.5:1	DMTR	55.58 ± 0.85	81.81 ± 1.87	436 ± 1.10
E5	1:1	BABZ	60.78 ± 1.74	88.21 ± 0.42	398 ± 2.45
E6	2:1	DMTR	44.35 ± 1.54	71.68 ± 1.52	508 ± 3.01
E7	1:1	DMTR	63.11 ± 1.83	91.38 ± 1.52	382 ± 2.36
E8	1.5:1	DMSO	64.57 ± 2.38	93.93 ± 2.35	376 ± 2.41
E9	2:1	BABZ	42.28 ± 1.64	65.73 ± 2.41	532 ± 1.97

2.2.11.2. Differential scanning calorimetry (DSC). The DSC analysis for pure enfuvirtide, blank ISFI formulation and optimized enfuvirtide ISFI formulation was carried out using a differential scanning calorimeter. For each scan, 1–2 mg of the sample was weighed and placed in a hermetically sealed aluminum pan. The samples were then heated at a heating rate of 10 °C/min under a nitrogen atmosphere (flow rate 20 mL/min), using an empty aluminum pan as the reference. All the samples were heated in the temperature range, 25–250 °C. The thermograms were used to determine onset, endset and peak glass transition temperatures (T_g).

2.2.11.3. Fourier transform infra-red (FTIR) spectroscopy. The conformational stability of enfuvirtide in the optimal formulation was determined by FTIR spectroscopy (Anderson, 2000). For solid samples, the sample was finely ground with KBr in an agate mortar. The powder was compressed into a pellet using a die-set and pellet press. The thin, transparent pellet was placed into the sample holder along with the collar. For liquid samples, a drop of liquid was placed between two NaCl plates to form a thin layer of liquid between them. Excess liquid was wiped off the edges and the sandwich plates were fitted into sample holder. The FTIR spectra of optimized enfuvirtide ISFI formulation was obtained in the frequency range of 500–4000 cm^{-1} and was compared with FTIR spectra of pure drug and blank ISFI formulation to assess any changes occurring to the drug on being formulated into a polymer solution.

2.2.11.4. CD (circular dichroism) spectroscopy. To compare any conformational changes incurred to the therapeutic polypeptide enfuvirtide, during formulation or during *in vitro* release studies, a drug solution (100 μg/mL) in phosphate buffer (pH 7.4) and a sample from the *in vitro* drug release studies was subjected to CD spectroscopy (Anderson, 2000). CD spectra were obtained with a Jasco J-810 spectropolarimeter equipped with a Peltier thermoelectric type temperature controller. Spectra were collected at 25 °C using a 0.1 cm cell over the wavelength range of 190–250 nm. A resolution of 0.1 nm and scanning speed of 10 nm/min with a 2 s response time were employed. Noise reduction and blank buffer subtraction were performed using standard analysis and wavelength analysis programs. The absorbance obtained was plotted against the respective wavelength for both the samples and the plots were compared for conformational stability.

2.2.11.5. Stability studies. The optimized ISFI formulation was subjected to stability studies by keeping them at accelerated and long term conditions of temperature and humidity (25 °C ± 2 °C/75% RH ± 5% RH; 5 °C ± 2 °C) for 6 months (ICHQ1F, 2006; WHO, 2009). Zero time samples were used as controls. Samples withdrawn at predetermined intervals (0, 1, 3 and 6 months) were analyzed for various performance parameters, *i.e.*, drug content, viscosity and drug release. The formulations were also visually inspected for any change in their physical appearance, *i.e.*, color, turbidity, odor, consistency, *etc.*

2.2.11.6. In vivo pharmacokinetic studies of enfuvirtide ISFI formulation. In vivo studies of enfuvirtide ISFI were carried out in male Sprague–Dawley rats weighing 150–200 g. All animal procedures were approved by the Institutional Animal Ethics Committee of the Panjab University, Chandigarh. The animals were randomly divided into 4 groups of 3 animals each to receive different treatments. Group I rats were administered with a single *s.c.* dose (562 μg/0.2 mL) of pure enfuvirtide solution in pH 7.4 phosphate buffer. Group II rats received a single *s.c.* dose of optimized ISFI formulation containing 562 μg enfuvirtide. Group III rats were administered a single *s.c.* dose of enfuvirtide 142.5 μg/0.2 mL pH 7.4 phosphate buffer and group IV rats were administered with a single *s.c.* dose of 0.2 mL ISFI without drug.

Blood (0.5 mL) from all animals was collected into PTFE tubes, containing EDTA as anticoagulant, from the retro-orbital cavity of the animals at pre-determined time interval, *i.e.*, at 0 (predose), 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 24, 32, 40, 48, 60 and 72 h after *s.c.* administration of enfuvirtide. The tubes were then centrifuged at 8000 × *g* for 15 min to separate the plasma. Plasma was transferred to a fresh PTFE tube and stored at −20 °C until analysis. Prior to analysis, extraction of enfuvirtide from animal plasma was performed by the addition of 50 μL of enfuvirtide containing plasma to 450 μL of ACN containing 1% TFA and 1% *n*-nonyl-*β*-D-glucopyranoside (ACN extraction mixture) in a PTFE microfuge tube (Lawless et al., 1998). This solution was vortexed for 15 s, then centrifuged at 8000 × *g* for 5 min. The supernatant was then diluted with HPLC water (supernatant–water, 40:60, v/v) for injection onto the HPLC column. Plasma samples from placebo treated animals were used as blank after similar treatment to account for any interference in the analysis of drug. All steps were performed at room temperature.

2.2.11.7. Determination of enfuvirtide in plasma by HPLC method. Concentrations of enfuvirtide in the plasma samples were analyzed by HPLC method as reported by Lawless et al. (1998). The method was validated for linearity, accuracy and precision. The values of LOQ (limit of quantitation) and LOD (limit of detection) of enfuvirtide were calculated using standard calibration curve as 3.3 σ/S and 10 σ/S, respectively, where S is the slope of the calibration curve and σ is the standard deviation of the response.

The equipment used for the analysis was a Waters® Alliance HPLC system with Waters® 1525 binary pump, 2707 autosampler, 2475 Multi-λ Fluorescence Detector fitted with 150 W xenon lamp, an 8 μL flow cell and Waters Empower™ 2 operating software. A Bondapack® C-18 column (250 × 4 mm i.d.) with a particle diameter of 10 μm and a pore size of 125 Å from Waters® was used. The mobile phase consisted of 0.1% TFA and 1% arginine hydrochloride; employing a 70% ACN–30% distilled water co-solvent for the organic buffer. Reverse phase separations were carried out using a 30–60% acetonitrile gradient run at the flow rate of 1 mL/min. Fluorescence detection was performed with excitation and emission wavelengths of 280 and 350 nm, respectively. Fluorescence detector gain was set at 1000, with an attenuation setting of 1 and employed a standard detector response time. The flow rate was 1 mL/min and the injection volume was 100 μL.

2.2.11.8. Pharmacokinetic data analysis. The pharmacokinetic parameters *viz.*, C_{max}, T_{max}, $T_{1/2\alpha}$, $T_{1/2\beta}$, K_a, K_e, AUC_{0-48} and AUC were calculated from plasma concentration *vs* time data using WinNonlin® version 5.2.1 software by compartmental analysis (2008; Ritschel, 1992). Mean values of AUC, C_{max}, $T_{1/2\alpha}$ and $T_{1/2\beta}$ were compared for group 1, group 2 and group 3 by using one-way ANOVA technique (Pharsight, 2008).

2.2.12. Biocompatibility study of enfuvirtide ISFI system

To determine the biocompatibility of enfuvirtide ISFI, nine mice were divided into three groups of three mice each, *i.e.*, positive control, test and negative control. The positive control group received enfuvirtide dispersed in pH 7.4 phosphate buffer, test group received ISFI with enfuvirtide and negative control group received ISFI without drug. All these animals were observed visually for any physical symptoms of inflammation. The inflammatory response to the implants was scored as: 0 (normal), + (mild), ++ (moderate), +++ (severe). Presence of redness, exudates, scratching or touch response was marked as R, E, S and T.

One mouse from each group was sacrificed on day 3, day 7 and day 14 after injection. The tissue surrounding the implant

were excised and fixed in 10% phosphate buffered formalin. The fixed sections were stained with hematoxylin and eosin. These were observed under optical microscope for acute inflammatory changes, foreign body reaction and fibrosis (Anderson and Langone, 1999; Park and Park, 1996).

3. Results and discussion

3.1. Solubility studies of enfuvirtide

It was observed that that enfuvirtide was practically insoluble or poorly soluble in most of the solvents including distilled water. However, it was found to be soluble in DMF (112.42 mg/mL) with comparatively lower solubility in DMSO, DMTR and BABZ. Enfuvirtide is a basic drug and its solubility is reported to increase with the increase in pH (EMEA, 2006; USFDA, 2010). Its solubility increased from 68.79 mg/mL in pH 7.4 phosphate buffer to 88.48 mg/mL at pH 8.5. Though, enfuvirtide has good solubility in DMF, it is not a suitable solvent for biological system due to its toxic and tissue irritating property (USFDA, 1997) and was, thus, ruled out. The selected solvent systems were DMSO, DMTR (60:40) and BABZ (70:30). As the drug was found to be sufficiently soluble in pH 7.4 phosphate buffer, this was selected as suitable media for drug release studies.

3.2. Micronization of enfuvirtide

It was observed from the particle size distribution graphs and photomicrographs of both un-micronized and micronized drug (graphs not shown) that micronization process significantly reduced the particle size of the drug. Mean particle size (d_{50}) for un-micronized drug was found to be 55.64 μm and 90% of particles (d_{90}) were equal to or below 175.43 μm whereas mean particle size (d_{50}) for micronized drug was found to be 4.58 μm and 90% of particles (d_{90}) were equal to or below 10.89 μm. The particles of un-micronized drug were also found to have non-uniform and wider range of size distribution (1.9–363 μm) as compared to micronized drug (0.55–45 μm), which presented uniform and normal distribution. The photomicrographs also revealed fine and uniformly distributed particles of micronized drug and larger non-uniformly distributed particles of un-micronized drug.

3.3. Preparation of enfuvirtide ISFI formulations

Four formulations, PF-1, PF-2, PF-3 and PF-4 were prepared using un-micronized drug by ultrasonication, micronized drug by ultrasonication, un-micronized drug by homogenization and micronized drug by homogenization, respectively. From the results of these formulations, micronized drug and homogenization method using Ultra Turrax were found to be suitable for preparation of enfuvirtide ISFI formulations.

3.4. Formula optimization of enfuvirtide ISFI system by design of experiment (DoE) method

Table 2 shows the values of response variables, Y_1 (percent drug released in 24 h), Y_2 (percent drug released in 48 h) and Y_3 (viscosity) obtained from batches prepared according to I-optimal response surface design.

In order to determine the significant design terms, their interactions and their effect on the response variables Y_1, Y_2 and Y_3, the design was evaluated by response surface linear model that was selected on the basis of model p-values, lack of fit test, adjusted R^2 and predicted R^2. Linear polynomial model equations were generated by ANOVA. Being close to 1.000, the values of R^2 for linear model indicated excellent fit of response surface polynomials to the response variable data. All the lack of fit values was found to be insignificant ($p > 0.05$) thus, indicating the validity of selected models. The closeness of adjusted R^2 and predicted R^2 to actual model R^2 also indicated the goodness of fit to the data. The model terms X_1 and X_2 were significant ($p < 0.0001$). Final first order polynomial equations for each response variable obtained from significant coefficient terms are given below:

$$Y_1 = 54.98 - 9.50X_1 + 3.51X_2 - 0.43X_2' \ldots \quad (6)$$

$$Y_2 = 81.39 - 11.19X_1 + 3.43X_2 + 0.25X_2 \ldots \quad (7)$$

$$Y_3 = 440.26 + 65.16X_1 - 21.06X_2 + 2.54X_2' \ldots \quad (8)$$

As the design involved one catagoric factor (solvent) as one of the axes, it was not possible to obtain contour or 3-D plots. Instead, interaction graphs were plotted to study the influence of significant model term X_1 (PLGA:Drug ratio) on different levels of factor X_2, *i.e.*, DMSO, DMTR and BABZ or *vice versa* in terms of responses Y_1, Y_2 and Y_3. Since, the 95% confidence interval bands of these interaction plots did not overlap with each other, significant difference existed between two design points. As, no interaction terms such as X_1X_2 were observed to be present in the model Eqs. (6)–(8), any significant interaction between the model terms is ruled out. Straight lines observed in the interaction plots indicated the goodness of fit of linear model. Overall, the above data obtained by model analysis indicates the suitability and significance of the selected design, factors, levels and responses.

3.5. In vitro enfuvirtide release from ISFI systems

Drug release profile of different formulations is shown in Fig. 1 as a plot of percent drug release *vs*. time. As seen in the graph, formulation E9 displayed lowest drug release, 42.28% (24 h) and 65.73% (48 h), whereas, formulation E2 displayed highest drug release, 68.36% (24 h) and 96.77% (48 h). The release study showed a biphasic pattern. The first phase of nearly 4 h indicated burst release of the drug that ranged from 16.55% (Formulation E9) to 40.45% (Formulation E2). In the case of an ISFI system, burst effect occurs due to fast diffusion of the drug during the lag time between the injection of liquid formulation and formation of the solid implant. Burst effect was found to be lower in the formulations prepared with high PLGA content because high polymer concentrations tend to increase the viscosity of the system and thus slowing down the removal of drug into the medium (Eliaz and Kost, 2000). The viscosity of formulation E9 was found to be higher (532 cP) than formulation E2 (352 cP). Another factor that may affect the burst release is the type of solvent used to prepare the ISFI system (Kranz and Bodmeier, 2007). It was observed that the effect of solvent was related to the viscosity or polymer content of the system. For instance, burst release of E2 (DMSO) was higher than E7 (DMTR) and E5 (BABZ), since, all these three formulations contain 1:1 PLGA:Drug ratio. Burst release of the formulations prepared with 2:1 PLGA:Drug ratio was found to be lowest but in the similar pattern, *viz*., DMSO > DMTR > BABZ. This may occur due to higher miscibility or hydrophilicity of DMSO in comparison to DMTR or BABZ with aqueous media. Therefore, when the drug-polymer solution prepared with DMSO is introduced in aqueous environment, the solvent is quickly removed from the polymer matrix into the surrounding release media, forming the implant. This implant, though, formed quickly will also carry some drug with the solvent into the release media leading to higher burst effect.

Following the initial protein burst within first 4 h, a continuous and constant increase in the release rate of all the protein formulations was observed over the next 44 h. Though, the drug release profile in this second phase appears to be similar and almost linear, the quantitative increment and variation between different formulations is still significant, as observed from the results of experimental design analysis in Section 3.4. So, drug release profile

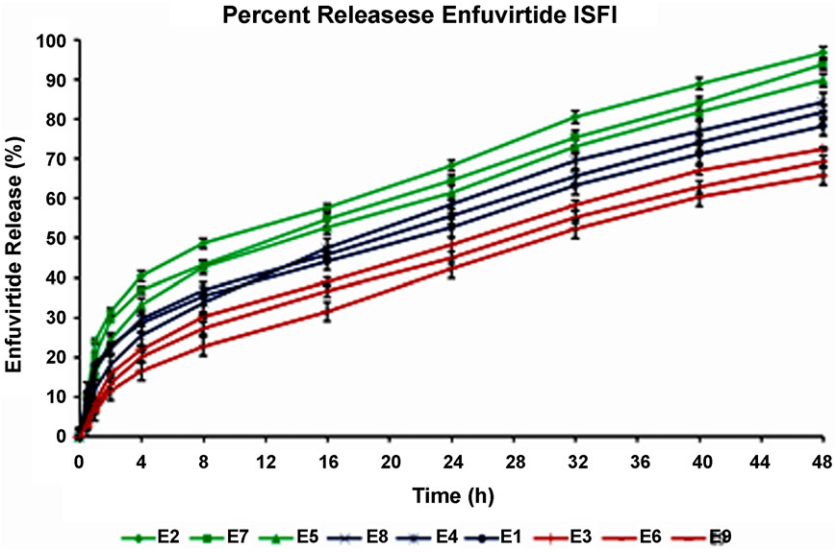

Fig. 1. Cumulative percent drug release profile of enfuvirtide ISFI formulations.

was also evaluated for 'goodness-of-fit' into various mathematical model equations to determine the release kinetics of the formulations. The R^2, k and n values of the model equations suggested that most of the formulations followed Higuchi and Peppas models. The results indicated drug release by diffusion, either by fickian or by anomalous transport depending up on the 'n' values of Peppas model ($0.5 < n < 1$).

As the release media diffuses in to the deeper core of the formulation, the polymer precipitates and forms various interconnecting channels and pores, through which the dissolved drug diffuses out into the external media (Brodbeck et al., 1999). The overall short duration of drug release (48 h) from the polymer matrix is because of the lower molecular weight of PLGA (6000 Da). Although, the polymer would not degrade in 48 h, but in case of low molecular weight PLGA, the chain scission process of the hydrolysable polymer starts immediately after its incubation in the release media. The process maybe further accelerated by using PLGA grade with higher glycolic acid content (PLGA 50:50) as it makes the system more hydrophilic. This phase is followed by degradation phase where bulk erosion of the polymer may add to the diffusion mechanism (Graham et al., 1999). The complete biodegradation period of the polymer is dependent upon the various factors such as molecular weight, hydrophilic–hydrophobic content, pH, end chain capping, temperature and solvent or media surrounding the implant (Ravi et al., 2008). More hydrophilic, low molecular weight PLGA based injectable formulations would degrade at a faster rate as compared to those with more hydrophobic and high molecular weight characteristics (Jalil and Nixon, 1990; Chen and Singh, 2005).

3.6. Determination of an optimized enfuvirtide ISFI formulation

The optimal formulation was searched within the studied experimental domain by numerical optimization technique. The constraints were set to the desired goals and entire experimental domain was searched for the compositions where the set constraints were met to the maximum. Table 3 presents the constraints set for numeric optimization, the resulting optimized solutions, formulation compositions with corresponding response and desirability values. The results obtained by numeric optimization represent the three best solutions for the three catagoric factors, DMTR, BABZ and DMSO. The solutions fulfill the target criteria of maximum release and minimum viscosity at low to intermediate levels of PLGA:Drug ratio (1.07:1–1.42:1). Finally, the formulation corresponding to solution 1 was selected as the optimum formulation that had the desirability of 0.649 and the values of response variables were in the desired range. The formulation contained 1.2:1 PLGA:Drug ratio and DMTR as solvent with 60% minimum release at 24 h, 88% maximum release at 48 h and 405 cP of minimum viscosity.

3.7. Validation of optimization results

The optimized formulation obtained by numeric optimization was validated for its performance by preparing all the three resulting formulations (EO1, EO2 and EO3) and determining their release and viscosity. Formulations EO1, EO2 and EO3 contained PLGA:Drug ratios 1.2:1, 1:1, 1.4:1 and solvents DMTR, BABZ and DMSO, respectively. Fig. 2 shows the drug release profile of the three formulations. The actual results obtained were compared with the predicted results. Table 4 shows the experimental values, predicted values and percent prediction error of response variables Y_1 (percent release at 24 h), Y_2 (percent release at 48 h) and Y_3 (viscosity) for EO1, EO2 and EO3.

The percent prediction error values for different response variables ranged between −3.43 and 1.10 and the overall percent error

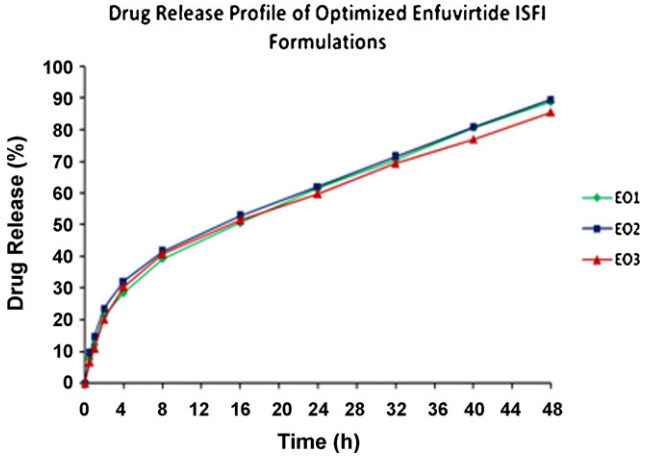

Fig. 2. Comparative percent release of enfuvirtide from the three optimized ISFI formulations.

Table 3
Numeric optimization for determination of optimal ISFI formulation of enfuvirtide.

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
Constraints						
A:PLGA:Drug	In range	1	2	1	1	3
B:Solvent	In range	DMSO	BABZ	1	1	3
Release 24 h (%)	In range	45	60	1	1	3
Release 48 h (%)	Maximize	75	96.77	1	1	3
Viscosity (cP)	Minimize	352	532	1	1	3

Number	PLGA:Drug	Solvent	Release 24 h	Release 48 h	Viscosity (cP)	Desirability
Solutions for 3 combinations of catagoric factor levels						
1	1.21:1	DMTR	60	88.06	405.41	0.649
2	1.07:1	BABZ	60	87.24	403.28	0.634
3	1.42:1	DMSO	60	86.60	408.83	0.604

Table 4
Comparative predicted *vs* experimental values of different response variables for optimized batches EO1, EO2 and EO3.

Batch no. (X_1, X_2)	Response	Predicted value	Experimental value	Percent prediction error
EO1 (1.2:1, DMTR)	Release 24 h (%)	60	61.58	−2.63
	Release 48 h (%)	88.06	88.89	−0.94
	Viscosity (cP)	405	407	−0.49
EO2 (1:1, BABZ)	Release 24 h (%)	60	62.06	−3.43
	Release 48 h (%)	87.24	89.61	−2.72
	Viscosity (cP)	403	409	−1.49
EO3 (1.4:1, DMSO)	Release 24 h (%)	60	59.86	0.23
	Release 48 h (%)	86.60	85.65	1.10
	Viscosity (cP)	408	412	−0.98

Overall percent prediction error (mean ± SD) = −1.26 ± 1.47.

was found to be −1.26 ± 1.47 indicating low variation between predicted and experimental results. From the drug release profile shown in Fig. 2, it was observed that all the three formulations presented almost similar release rate and pattern. However, for practical purpose, the one with highest release and desirability (EO1) was selected as the optimized and validated formulation.

3.8. Evaluation of optimized enfuvirtide ISFI formulation

3.8.1. Rheological studies

The rheological behavior of the optimized formulation EO1 is shown in Fig. 3(a) and (b). Viscosity values ranged from 425 to 389 cP over increasing shear rate from 1 to 100 s^{-1}, indicating slight decrease in viscosity on increasing shear rate. The average viscosity was found to be 407.03 ± 10.82 cP. Plot of shear stress *vs* shear rate is shown in Fig. 3(b) which also shows power law equation and trend line obtained from log shear stress and log shear rate. Application of the power law model to the rheological properties of each formulation enabled the calculation of the consistency (k) and flow index (n).

The value of consistency index and flow index were found to be 12.85 and 0.764 for EO1. The R^2 value for the curve fitting line was 0.9830 indicating goodness of fit for power law. The flow index value of 0.764 indicated the shear thinning or pseudoplastic behavior of the formulation ($n < 1$). This shear thinning property is useful for good flowability during injection of the formulation from a syringe. Thus, from these results it was construed that the formulation EO1 displayed sufficient consistency and good flowability.

3.8.2. Stability of enfuvirtide in ISFI formulation by DSC

Fig. 4(a)–(c) shows DSC thermograms of blank ISFI (without drug), enfuvirtide and enfuvirtide ISFI formulation EO1, respectively. Enfuvirtide gives a sharp endothermic peak at 96.25 °C with an onset temperature of 64.49 °C. Thermogram of blank formulation shows a broad endothermic peak at 50.21 °C indicating the amorphous nature of the polymer PLGA 50:50. This peak also confirms the glass transition temperature of PLGA 50:50, which is approx. 50 °C. Enfuvirtide ISFI formulation shows endothermic peaks for both PLGA and enfuvirtide at 50.11 °C and 95.25 °C, respectively. This revealed that no chemical interaction occurred

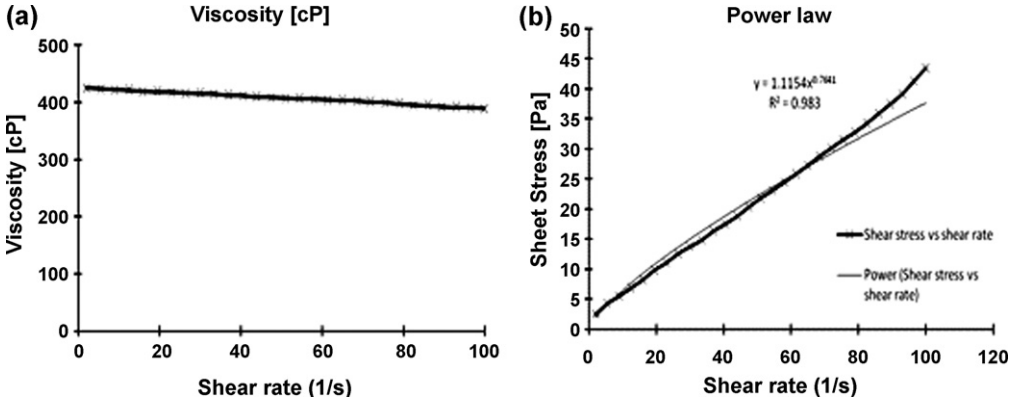

Fig. 3. Rheograms of enfuvirtide ISFI formulation EO1 (a) Shear rate *vs* viscosity (b) Shear stress *vs* shear rate.

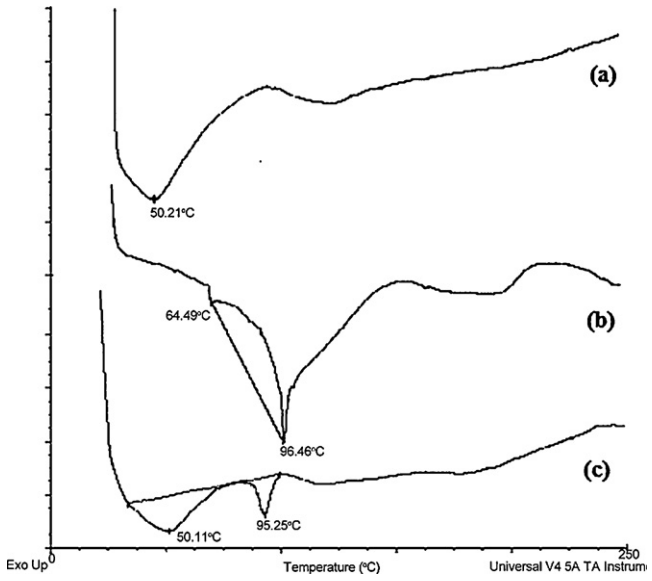

Fig. 4. DSC thermogram of (a) blank formulation, (b) enfuvirtide, (c) enfuvirtide ISFI formulation.

between the drug and polymer indicating compatibility and stability of the drug and the polymer in the formulation.

3.8.3. Stability of enfuvirtide in ISFI formulations by FTIR

Fig. 5(a)–(c) depicts FTIR spectra of blank ISFI, enfuvirtide and enfuvirtide ISFI formulation EO1, respectively. Enfuvirtide shows a prominent peak at 1654.2 cm^{-1} that is within the range characteristic for proteins (1600–1700 cm^{-1}). Therefore, the optimized

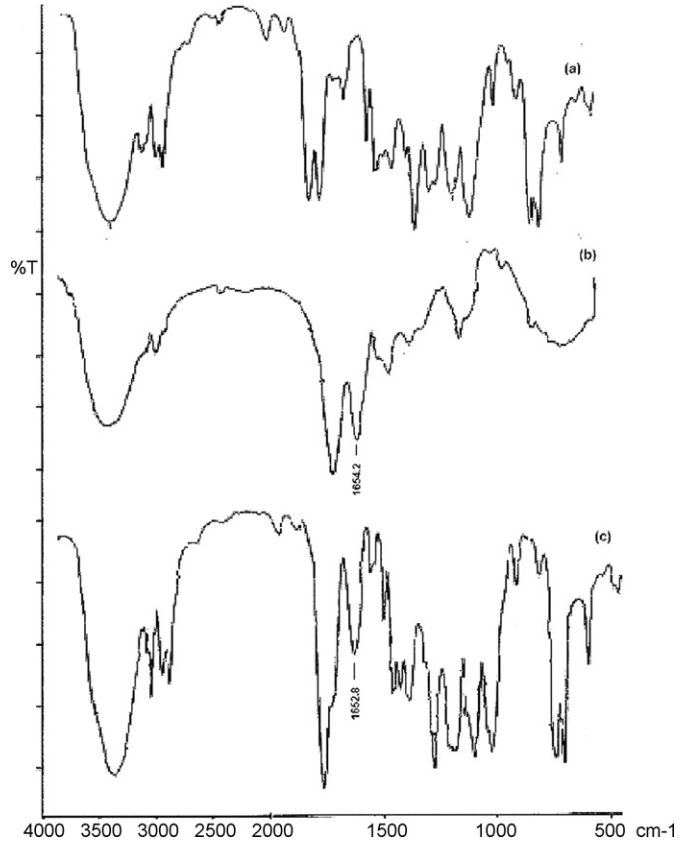

Fig. 5. FTIR spectra for (a) blank ISFI, (b) enfuvirtide, (c) enfuvirtide ISFI formulation EO1.

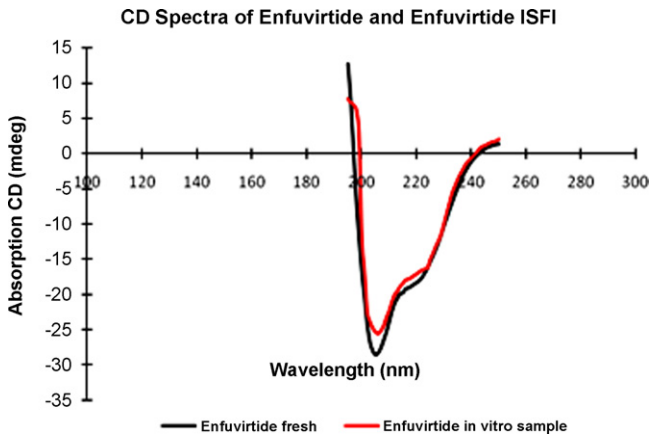

Fig. 6. CD spectra of freshly prepared enfuvirtide solution and *in vitro* release sample of enfuvirtide ISFI formulation withdrawn after 48 h.

formulation was subjected to FTIR analysis to check the presence of peak in this region. It was found that the enfuvirtide ISFI formulations showed absorption peaks at 1652.8 cm^{-1} indicating the conformational stability of enfuvirtide in formulation. An FTIR spectrum of blank formulation without drug was also obtained in order to compare the peaks arising due to the vehicle of the formulation. Therefore, the results indicated good stability of the protein drug in the formulation. In addition, since no marked shift in the major peaks of drug and polymer was found, it was concluded that the drug was stable in the formulation. It also indicated that the drug was present as a physical mixture in the formulation and did not interact chemically with the polymer.

3.8.4. Conformational stability of enfuvirtide in ISFI system by CD spectroscopy

CD spectroscopy is a specialized form of absorption spectroscopy, which is extremely sensitive to the conformation of the protein. The presence of ordered secondary structures can be evaluated from the far UV CD spectrum between 250 and 190 nm, which is dominated by amide bond absorption. Fig. 6 shows the absorption plotted against respective wavelengths for pure enfuvirtide freshly dissolved in pH 7.4-phosphate buffer (100 μg/mL) and sample collected from *in vitro* release medium of enfuvirtide ISFI formulation EO1 after 48 h release study.

The CD spectra of both freshly prepared enfuvirtide solution and *in vitro* release sample show maxima at 195 nm and minima at 205 as well as 206 nm, respectively. The minima indicative of α-helicity shows low helical content in enfuvirtide. According to the CD spectroscopy measurements carried out by Veiga et al., the α-helix content of enfuvirtide was only 8% in aqueous buffer (Veiga et al., 2004). Enfuvirtide remains essentially in a random coil conformation in aqueous solutions and does not undergo random coil-to-helix transformation in both aqueous and lipid environments. The presence of large number of hydrophobic amino acid residues in the sequence of enfuvirtide would cause it to fold forming globular structures improving its solubility while protecting the more hydrophobic residues from the solvent that stabilizes the structure in solution. As shown in Fig. 6, the plots between wavelength and absorption for the two samples are nearly superimposable with minor weakening of CD spectrum, indicating conformational stability and absence of denaturation of enfuvirtide in the ISFI formulation during *in vitro* studies.

3.9. Stability studies

No physical change in appearance, odor or consistency of the formulations was observed after 0, 1, 3 and 6 months of storage

Table 5
Evaluation parameters for enfuvirtide ISFI formulation stored at accelerated and long-term stability conditions.

Time (months)	25 °C ± 2 °C/60% ± 5% RH		5 °C ± 2 °C	
	Residual drug (%)	Viscosity (cP)	Residual drug (%)	Viscosity (cP)
0	100	394 ± 6.25	100	388 ± 5.70
1	98.29 ± 0.1.1	385 ± 4.51	99.65 ± 0.86	383 ± 3.98
3	97.72 ± 1.9	379 ± 5.49	98.72 ± 1.4	379 ± 4.76
6	95.21 ± 1.2	371 ± 6.76	97.51 ± 1.6	375 ± 2.44

Table 6
Pharmacokinetic parameters determined for groups I, II, III animal.

Group	AUC[a] (h*ng/mL)	K_a[b] (h^{-1})	K_e[c] (h^{-1})	VD[d] (mL)	CL[e] (mL/h)	T_{max}[f] (h)	C_{max}[g] (ng/mL)	$T_{1/2\alpha}$[h] (h)	$T_{1/2\beta}$[i] (h)
I	12793.00	0.0827	0.0837	524.31	43.93	12.01	391.77	8.38	8.27
SE	2471.54	3.59	3.65	22936.15	8.49	2.21	33.42	364.36	360.40
CV%	19.32	4352.12	4360.76	4374.49	19.34	18.37	8.53	4347.77	4356.41
II	14797.97	0.8175	0.0266	1427.61	37.98	20.36	229.06	8.47	26.05
SE	787.06	0.0116	0.0040	150.35	2.02	0.65	4.11	1.21	3.95
CV%	5.32	14.30	15.18	10.53	5.32	3.24	1.79	14.28	15.17
III	2302.51	0.1733	0.1719	354.78	61.02	5.79	146.25	3.99	4.03
SE	467.31	14.78	14.67	30225.18	12.40	0.87	15.53	340.85	343.62
CV%	20.30	8531.29	8535.04	8519.33	20.32	15.11	10.62	8522.77	8526.52

[a] Area under the curve.
[b] Absorption rate constant.
[c] Elimination rate constant.
[d] Volume of distribution.
[e] Clearance.
[f] Time for maximum plasma concentration.
[g] Maximum plasma concentration.
[h] Absorption half life.
[i] Elimination half life.

at both 25 °C ± 2 °C/60% RH ± 5% RH and at 5 °C ± 2 °C conditions. Table 5 shows drug release profile of the optimized enfuvirtide ISFI formulations EO1a, EO1b, EO1c and EO1d stored at 25 °C ± 2 °C/60% RH ± 5% RH and 5 °C ± 2 °C analyzed at 0, 1, 3 and 6 month duration. Table 5 also shows the percent residual drug and viscosity of the stored formulations analyzed at specified time point. It was observed that drug release increased as the storage time at 25 °C ± 2 °C/60% RH ± 5% RH and 5 °C ± 2 °C increased in the order 0, 1, 3 and 6 months. The maximum drug release from the formulations stored at 5 °C ± 2 °C was 93.79% at 6 months and 98.36% for formulations stored at 25 °C ± 2 °C/60% RH ± 5% RH. The difference was within ±5% variation range, which is not significant. A slight increase in the burst effect was also observed in the formulations stored at accelerated conditions. The increase in the drug release could be attributed to degradation of the polymer during the storage. The increase was however, not very high and was acceptable considering the conditions and duration of the storage.

As seen in Table 5, the percent residual drug in the formulations stored at 5 °C ± 2 °C decreased from 100% to 97.51% after 6 months and decreased to 95.21% after 6 months for formulations stored at 25 °C ± 2 °C/60% RH ± 5% RH. The decrease in the drug content of the formulations was not more than 5% for any condition or duration indicating the stability of the drug in the formulations. The 5% decrease in the drug content could have occurred due to the degradation tendency of a protein molecule at long term long term elevated temperature storage. Similarly, the variation in the values of viscosity was also acceptable and passed the 5% criteria for both the conditions. These results indicate that the optimized enfuvirtide ISFI formulation would maintain its stability if stored at refrigerated conditions (2–8 °C) for at least 6 months.

3.10. *In vivo pharmacokinetic studies of enfuvirtide ISFI formulation*

The plasma concentration–time profiles of enfuvirtide in the individual animals including the respective mean and standard deviation (±SD) values and following each of the respective treatment for pure drug (562 μg/0.2 mL) as group I, optimized enfuvirtide ISFI formulation EO1 (562 μg/0.2 mL) as group II and pure drug (142.5 μg/0.2 mL) as group III, are graphically depicted in Fig. 7. All the formulations were administered by *s.c.* route. Pharmacokinetic data analysis and modeling was accomplished by fitting the 1 compartment first order kinetics into the plasma concentration data of enfuvirtide in rats.

Table 6 shows different pharmacokinetic parameters (AUC, K_a, K_e, VD, CL, T_{max}, C_{max}, $T_{1/2\alpha}$ and $T_{1/2\beta}$) determined from the plasma concentration-time data of the three animal groups by using WinNonlin® software. The method used by the software to calculate the pharmacokinetic parameters was Gauss–Newton (Levenberg and Hartley) method. The correlation values for predicted and actual concentrations were 0.9151, 0.9936 and 0.8949. The AUC_{0-48} for group I, II and III computed by trapezoidal rule was found to be 11202.4, 11472.8 and 2178, respectively.

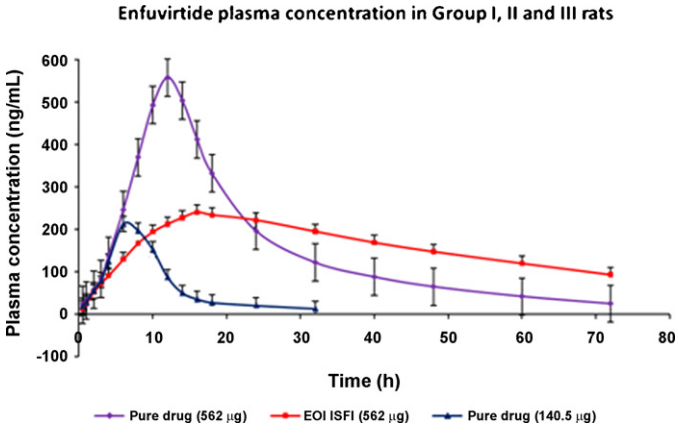

Fig. 7. Plasma concentration profiles of enfuvirtide in groups I, II and III animals.

Table 7
Comparison of AUC, $T_{1/2\alpha}$, $T_{1/2\beta}$ and C_{max} for groups I, II and III by ANOVA.

Pharmacokinetic parameter		Treatment (between columns)	Residual (within columns)	Total	*p*-Value
AUC	SS[a]	270207000	41678100	311885000	0.0024
	DF[b]	2	6	8	
	MS[c]	1.35E+08	6946350		
$T_{1/2\alpha}$	SS	39.3506	1493630	1493670	0.9999
	DF	2	6	8	
	MS	19.6753	248938		
$T_{1/2\beta}$	SS	818.986	1487870	1488690	0.9983
	DF	2	6	8	
	MS	409.493	247978		
C_{max}	SS	93612.1	8249.82	101862	0.0005
	DF	2	6	8	
	MS	46806.1	1374.97		

[a] Sum of squares.
[b] Degree of freedom.
[c] Mean squares.

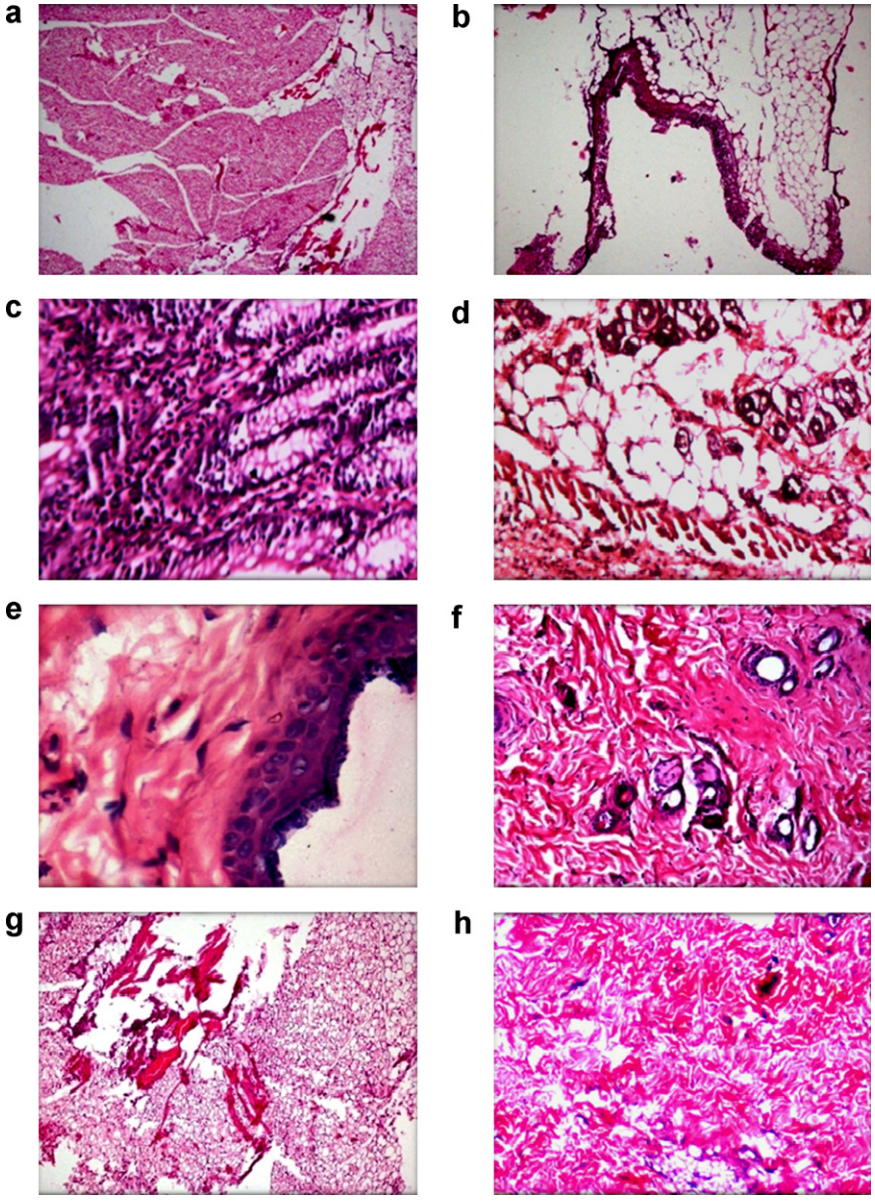

Fig. 8. (a) Transverse section of normal subcutaneous tissue of rat. (b) Transverse section of rat subcutaneous tissue on day 3 of blank ISFI administration. (c) Transverse section of rat subcutaneous tissue on day 3 of pure enfuvirtideadministration. (d) Transverse section of rat subcutaneous tissue on day 3 of enfuvirtide ISFI administration. (e) Transverse section of rat subcutaneous tissue on day 7 of blank ISFI administration. (f) Transverse section of rat subcutaneous tissue on day 7 of pure enfuvirtide administration. (g) Transverse section of rat subcutaneous tissue on day 7 of enfuvirtide ISFI administration. (h) Transverse section of rat subcutaneous tissue on day 14 of enfuvirtide ISFI administration.

Table 7 shows one-way ANOVA results of different pharmacokinetic parameters applied to determine their significance level. The results were considered as significant if $p < 0.05$. The values for treatment and residual sum of squares, degree of freedom, mean squares and their respective p values for each group are given in the table. According to these results, AUC, C_{max}, and T_{max} were found to be significant whereas $T_{1/2\alpha}$ and $T_{1/2\beta}$ were not.

From the *in vivo* pharmacokinetic studies, it was observed that group III samples presented with the lowest C_{max} (212.8 ng/mL) and T_{max} (6 h). This group received pure enfuvirtide equivalent to a conventional single dose. Group I showed the highest C_{max} and T_{max}, *viz*., 557.60 ng/mL and 12 h, respectively. This group received pure enfuvirtide equivalent to a 48 h depot dose. However, in both the cases, the C_{max} was not maintained for long time. After 48 h, the plasma concentration falls to 64.6 ng/mL in group I animals and to non-detectable quantities in and group III animals. The C_{max} and T_{max} values of group II animals were found to be 239.80 ng/mL and 16 h, respectively. The C_{max} value in group II was close to the C_{max} of group I indicating the safety of the ISFI formulation. It was observed in the profile of group II that once the concentration equivalent to that of a single *s.c.* dose is reached, it is maintained for longer duration and was 147.3 ng/mL at 48 h and 93.2 ng/mL at 72 h. These results indicated that the optimized ISFI formulation maintained nearly constant steady state plasma concentration and remained within the effective levels even after 72 h, whereas the plasma concentration of pure enfuvirtide either increased to unnecessary high levels in group I or reduced to ineffective levels in group III in less than 48 h.

Thus, the *in vivo* pharmacokinetic study results suggested that it was possible to effectively control the enfuvirtide release and maintain the therapeutic concentration in rat model for 48 h. These results were achieved when four conventional doses of enfuvirtide were combined into a single *s.c.* dose of the optimized enfuvirtide ISFI formulation (EO1). This delivery system would be able to replace four conventional, *s.c*., injections of enfuvirtide (48 h therapy). Since, considerable amount of drug was detected in the plasma samples up to 72 h, the developed ISFI system can potentially be modified to replace six conventional dose injections (72 h therapy) by adjusting the dose and controlling the *in vitro* drug release.

3.11. Biocompatibility study of enfuvirtide ISFI system

During the physical observation, it was observed that there was a moderate inflammatory reaction with redness in all the rats of group I and group II but mild in the animals of group III that were administered placebo or ISFI without drug. It was observed that the animals of group I showed maximum inflammation followed by group II and minimum in group III. It could be interpreted from the results that ISFI formulation provided some protection from the inflammatory response as most of the drug is entrapped in the implant with only small amount of pure drug actually interacting the tissue at implant site at any point of time. These findings suggest the irritant nature of the protein drug enfuvirtide and confirms to the previous reports in literature.

3.11.1. Histopathological studies

After the physical observation, the biocompatibility of enfuvirtide ISFI was confirmed by the histopathological studies. Fig. 8(a)–(d) shows the transverse sections of normal *s.c.* tissue of rat, the *s.c.* tissue surrounding the blank ISFI, tissue surrounding the injection site of pure enfuvirtide and tissue surrounding enfuvirtide ISFI formulations. Only a few macrophages were found in the normal rat tissue. High macrophage accumulation or injury was visible in the blank, drug containing formulation treated tissues and pure drug treated tissue. This is because of the body's reaction to the foreign body invasion by the implant. As seen in Fig. 8(e)–(g) on day 7, the number of inflammatory cells found in the tissue had reduced in the entire tissue samples. Results for tissue sample surrounding enfuvirtide ISFI formulation that was obtained on day 14 after administration are shown in Fig. 8(h) wherein, very less macrophages were found. This shows that the formulation was biocompatible as it initiated only normal inflammatory response that subdued gradually within 15 days of administration.

4. Conclusions

Thus, from the above studies it was concluded that the developed and optimized enfuvirtide ISFI short term formulation could satisfactorily and effectively serve the purpose of extending the protein release for a pre-determined and specified duration of 48 h. Further, *in vivo* pharmacokinetic and biocompatibility studies revealed that sufficient plasma concentration was maintained during the duration of protein release and the formulations were biocompatible. Since, the concentration continued to be maintained up to 72 h, effective release of enfuvirtide may also be extended further, up to 72 h after minor modification in the formulation. Further, detailed pharmacodynamic studies including safety, efficacy and toxicity products. Therefore, these studies may be taken up as a subsequent research project.

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