Pharmaceutical Development and Technology

ISSN: 1083-7450 (Print) 1097-9867 (Online) Journal homepage: http://www.tandfonline.com/loi/iphd20

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To cite this article: Behnoush Alizadeh, Nika Bahari Javan, Hamid Akbari Javar, Mohammad Reza Khoshayand & Farid Abedin Dorkoosh (2017): Prolonged injectable formulation of Nafarelin using in situ gel combination delivery system, Pharmaceutical Development and Technology, DOI: 10.1080/10837450.2017.1321662

To link to this article: http://dx.doi.org/10.1080/10837450.2017.1321662

Accepted author version posted online: 21 Apr 2017.

Prolonged injectable formulation of Nafarelin using *in situ* gel combination delivery system

Behnoush Alizadeh [1], Nika Bahari Javan [1], Hamid Akbari Javar[1], Mohammad Reza khoshayand[2], Farid Abedin Dorkoosh [*1,3]

[1]*Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Science, Tehran, Iran*

[2]*Department of Drug and Food Control and Pharmaceutical Quality Assurance Research Center, Faculty of Pharmacy, Tehran University of Medical Science, Tehran, Iran*

[3]*Medical Biomaterial Research Center (MBRC), Tehran University of Medical Science, Tehran, Iran*

Corresponding author:

Name: Farid Abedin Dorkoosh

Address: Pharmaceutical Products Technology Units Incubator, No. 1462, Kargar Ave., Tehran-Iran

Tel/Fax: +98 21 88009440

E-mail address: dorkoosh@tums.ac.ir

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Abstract

The principal purpose of the present study was to prepare and characterize a complex drug delivery system consisting of Nafarelin- poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanoparticles (NPs) in combination with sodium alginate/ poloxamer 407 *in situ* gel. Nafarelin loaded PHBV NPs were prepared via double emulsion solvent evaporation technique. Box-Behnken Response Surface Methodology was utilized to optimize NPs. Mean particle size, polydispersity index (PDI), entrapment efficiency (EE) and drug loading (DL) of the optimized NPs were measured. Incorporation of Nafarelin within NPs were proven by DSC. The combination delivery system (CDS) was prepared by adding Nafarelin loaded PHBV NPs to sodium alginate/ poloxamer 407 solution followed by physical mixing. Morphological properties of Nafarelin loaded PHBV NPs and CDS were evaluated by SEM. Rheological properties were employed to investigate the effects of alginate concentration on sol-gel transition temperature. The release profile of Nafarelin from both PHBV NPs and CDS were individually assessed. The cumulative release percentage from CDS was significantly lower than Nafarelin released from PHBV NPs. Based on the favorable results in this study, the CDS consisting of sodium alginate/ poloxamer 407 loaded with PHBV NPs could be a promising candidate for designing a long lasting formulation of Nafarelin.

Keywords: Nafarelin, Poly (3-hydroxybutyrate-co-3-hydroxyvalerate), Sodium alginate, Poloxamer 407, Combination delivery system

Introduction

Gonadotropin releasing hormone (GnRH) analogues are synthetic peptides that interact with GnRH receptor and cause the secretion of pituitary hormones, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Since 1981, GnRH analogues have been used in treatment of precocious puberty, prostate and breast cancer, endometriosis, severe cases of congenital adrenal hyperplasia and also *in vitro* fertilization (IVF) (Partsch 2002; Hayden 2008; Berberoğlu 2009). Various dosage forms of GnRH analogues including intranasal spray, three-monthly/ monthly/ daily injections, and implants are introduced to the market now (Okada 1994).

Nafarelin is a synthetic analogue of naturally existing GnRH administrated as a common treatment of central precocious puberty (CPP) (Lin 1986; Miller 2010), endometriosis (Henzl 1990; Zhao 1998), and also for ovary stimulation in *in vitro* fertilization (IVF) (Martin 1994). Currently, the only available dosage form of Nafarelin in the market is a metered spray pump (*Synarel*) that delivers 200 μg of Nafarelin per dose (Valle 2003). The recommended daily dose of Nafarelin in central precocious puberty is 1600 μg and may increase to 1800 μg/day. It is obvious that the multiplicity of drug administration can be distressing for patients in the long run and it occasionally leads to forgetting one or more doses of the spray which may eventually lead to failure of the treatment. Additionally, long term use of nasal sprays can cause nasal irritation and rhinitis in patients which will inevitably force patients to stop taking the drug (Pujara 1995; Dondeti 1996). Another disadvantage is that the inhalation dose depends on the pumping technique and breathing of the patient. Hence, fluctuations may lead to underuse or overuse of the medication (Behl 1998; Ritthidej 2011). Moreover, the device must be clean everyday otherwise clogged tip may occur which results in lowering the amount of medication absorbed by the patient (Valle 2003). These difficulties could be overcome by developing a new prolonged

injectable formulation of Nafarelin. This may accompany with various benefits for patients such as less frequency of using, less local adverse effects and better control of the drug's concentration level (Sudhakav 2013).

One of the common strategies to sustain the release profile of peptides and proteins is nanoparticles drug delivery systems (Tan 2010). NPs have been recommended as valuable carriers for protection of peptides and proteins from adverse degradation and they also display great stability and high loading capacity (Mohanraj 2006; Mohanraj 2007). The most extensively used polymers are polylactic-co-glycoloic acid (PLGA), polylactic acid (PLA), polycaprolactone (PCL), and polyalkyl cyanoacrylates (Kumari 2010; Pal 2011). Despite the wide usage of the aforementioned polymers in designing long-acting formulations of various therapeutic agents, there are still some issues remained unresolved. One of these problematic issues is swift degradation of PLGA which may lead to creation of an acidic environment. Aggregation and denaturation of peptides and proteins are the undesirable result of this issue which can cause immunogenicity. Crystalline residues which are remained even after several years due to slow degradation of some polymers like PLA is another problem worth noticing (Bahari Javan 2016). Finally, high adhesiveness of cyanoacrylates, which results in both low elasticity and low resistance to chemical and physical attack, has limited the application of the aforementioned polymers (Lim 2015). In order to eliminate the disadvantages of widely used polymers, we have selected an alternative polymer named PHBV. PHBV is a biocompatible (Fukada 1986), biodegradable (Koosha 1989) and non- toxic (Pouton 1996) polymer. Also the lower degradation rate of PHBV compared to PLGA and the higher degradation rate compared to PLA solves the issues outlined above (Kose 2005).

In situ gel forming systems have received significant attention over the past two decades. These systems undergo sol- gel transition after administration. Different mechanisms such as pH change (Srividya 2001; Wu 2007), temperature modulation (Wei 2002; Escobar-Chávez 2006) and ionic exchange (Ludwig 2005) can cause the gel formation. No study could be found for prolonged formulations of Nafarelin based on CDS in literatures. This is the first study in which a nanoparticulate carrier based on PHBV were loaded in *in situ* gel based on poloxamer 407. Poloxamer 407 could prepare a thermo responsible gel which is a system in a liquid form in room temperature but it converts into a gel form in body temperature. Poloxamers have been widely used in formation of *in situ* gels due to various advantages such as predictable sustained release and the ease of administration, which improves patient compliance and comfort (Dumortier 2006; Escobar-Chávez 2006; Simões 2012).

Further to the favorable results achieved in this study, it seems that formation of *in situ* gel CDS could pave the way for preparing long lasting formulations of various peptides and proteins.

Materials and Methods

Materials

Nafarelin acetate (CAS: 86220-42-0) was purchased from Henan New Sensation Chemical Co LTD, China. Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) was obtained from Ningbo Tianan Biologic Materials Co LTD, China. Poly Vinyl Alcohol (PVA) was obtained from Aldrich, Germany. Choloroform, Poloxamer 407 (Pluronic F127) and Sodium Alginate

were all purchased from Sigma, Germany. All other reagents were analytical grade and were purchased from Merck, Germany.

Methods

Design of experiments (DoE)

Design expert (version 6.0.10, stat-Ease, Inc., Minneapolis, MN) was employed to evaluate the effects of three quantitative independent parameters, including PVA concentration, drug concentration and PHBV concentration on EE%, DL% and size of NPs in 17 separated runs. The independent variables mentioned above, were chosen according to preliminary studies based on literatures (Cun 2011; Nabi-Meibodi 2013). Each run was prepared and analyzed 3 times. The range of PVA, Nafarelin and PHBV were 0.25- 3%, 400- 2000 µg/ml and 0.1- 1.5%, respectively.

The responses achieved by the optimum formulation based on optimization studies were practically compared with the responses predicted by a software – illustrated below.

Optimization process

The acquired results were investigated by ANOVA to identify the significance of the models. The final optimized formula was theoretically obtained based on maximum EE%, maximum DL%, and minimum particle size.

Preparation of Nafarelin loaded PHBV NPs

Double emulsion solvent evaporation technique ($W_1/O/W_2$) was utilized to prepare Nafarelin loaded PHBV NPs (Nicoli 2001; Khoee 2012; Bahari Javan 2016). In this method, an aqueous solution of Nafarelin (300 µl) was emulsified in 1 ml solution of PHBV (0.78%) in chloroform under probe sonicator (Amplitude: 50, Pulse: 0.5) for 1 minute. The resulting emulsion (W_1/O) was gradually dispersed in the second aqueous phase containing 5 ml PVA (1.65%) as an emulsifier to form double emulsion ($W_1/O/W_2$). The acquired emulsion was stirred for 4 hours to remove the organic solvent. Afterwards, the final emulsion was centrifuged for 10 minutes at 2000 rpm to remove microparticles. Additionally, the emulsion was centrifuged for 20 minutes with 14000 rpm to harvest the NPs and they were subjected for determination of DL% and EE%.

Dynamic light scattering (DLS)

Dynamic light scattering (DLS) (Malvern instrument, Worcestershire, UK) was used to determine the mean particle size, polydispersity index (PDI) and zeta potential of Nafarelin NPs. The DLS measurements were performed with diluted suspension of NPs at 1:10 v/v - water dilution. All the analyses were performed with a wavelength of 436 nm at 25°C by a detector at angle of 90°. DLS data was measured in triplicate (n=3).

Drug Loading (DL %) and Entrapment Efficiency (EE %)

In order to calculate the DL% and the EE% of the NPs, indirect method was utilized. In indirect method, the Nafarelin concentration in the supernatant was quantified by reverse phase HPLC

(RP-HPLC) (Agilent Technologies model 1260 Infinity) and then it was subtracted from the initial amount of peptide to determine the DL% and the EE%. A reversed phase column (C18AM-302, 5micrometer, 150×4.6 mm ODS-AM) which was protected by pre-column (5 micrometer, 4.0×4.6 mm ODS-AM) was utilized at room temperature. Chromatographic data were evaluated by Agilent ChemStation software (B.04.02). According to the method described by Richard A. Kenley, with minor modification, the flow rate and UV wavelength were 1 ml/min and 225 nm, respectively and a 40:60 mixture of acetonitrile and buffer plus 1.73ml triethylamine (TEA) (pH= 2.3) was used as a mobile phase (Kenley 1987). The mobile phase was filtered through Phenex™ Teflon (PTFE) filter membrane (0.45 um). For each run 20 microliter of supernatant was injected. EE% and drug DL% were calculated according to the following equations:

EE (%) = Amount of drug in the NPs/ Amount of drug fed into the system × 100 Equation 1

DL (%) = Amount of drug in the NPs/ Weight of NPs × 100 Equation 2

Scanning electron microscopy (SEM)

Morphological assessment of prepared NPs was examined by scanning electron microscopy (FESEM, S4160, and Hitachi, Japan). One drop of fresh NPs suspension was placed on a slide. After drying, the sample was coated with a fine layer of gold to facilitate electricity conduction. The images of the sample were produced with an accelerating voltage of 10 kV and a working distance of 14.21 mm.

Differential scanning calorimetry (DSC)

The thermal analysis was carried out using a differential scanning calorimeter (DSC- 823, Mettler Toledo, Switzerland). Thermograms of the different samples (NPs, pure Nafarelin and PHBV) were obtained using STARe software (version 9.01). The weighted samples (9.5-11 mg) were loaded in aluminum pans and heated from -30°C to 210°C at a heating rate of 10°C/min, using nitrogen flow as the purge gas.

In vitro Nafarelin release from optimized PHBV NPs

The release study of the optimized formula was performed using dialysis bag (cut-off 12 kDa). Initially, NPs were dried using lyophilizer Multi-Tainer (FTS Systems, INC). 25 mg of lyophilized NPs was introduced into dialysis bag and fully immersed in 10 ml phosphate buffered saline (PBS) (pH= 7.4). The release of free Nafarelin was carried out with the same method to prove that dialysis bag had no retaining effect on peptide release. During the experiment, samples were shaken horizontally at 37±1°C in an incubator shaker at 200 rpm. At certain time intervals, 0.5 ml of the sample was removed for analysis and the medium was replaced with the same quantity of fresh PBS to preserve the sink condition. The withdrawn samples were subjected to RP-HPLC for further analysis.

Gel preparation

Cold method was utilized to prepare poloxamer solution (14%). Briefly, the weighted amount of poloxamer 407 was dispersed in deionized water (4-8°C) under continuous stirring. The partially

dissolved poloxamer solution was kept at 4°C for further 24 h until a clear solution was obtained. Alginate sodium was dissolved in distilled water (70-80°C) to achieve the appropriate concentration level. The alginate solution was stored in refrigerator. Poloxamer 407 and alginate solutions were mixed at equal volume ratios. The formulations consisted of 14% poloxamer (F_1), 14% poloxamer with 0.1% sodium alginate (F_2) and 14% poloxamer with 0.3% sodium alginate (F_3).

Rheological properties

The viscoelastic properties and the sol-gel transition temperature of each formulation (F_1, F_2 and F_3) were investigated by Anto Paar Physica MCR 301 rheometer (Anton Paar Company, Austria). The storage (G') and loss (G") modulus were evaluated over temperature range of 15-50°C at a heating rate of 1°C min^{-1}, at a fixed frequency of 1 HZ and a maximum strain amplitude of 0.1 pa. The temperature, at which the storage modulus and loss modulus curves intersect, was considered as the gelation temperature. The influence of adding a mucoadhesive to poloxamer on gelation temperature was evaluated on F_2 and F_3 formulations.

Preparation of CDS

In order to prepare CDS, optimized Nafarelin loaded PHBV NPs were added to *in situ* forming gel in ratios of 0.5:1, 1:1, and 2:1 w/w with the continuous stirring.

Scanning electron microscopy (SEM)

Morphology of Nafarelin PHBV NPs loaded in optimized *in situ* gel formulation was characterized by scanning electron microscopy (FESEM, S4160, and Hitachi, Japan). One drop of sample was placed on a slide and after drying, the sample was coated with a fine layer of gold to facilitate electricity conduction. The sample was imaged with an accelerating voltage of 7 kV and a working distance of 9.7 mm.

Fluorescence spectroscopy

Fluorescence spectroscopy (LS 55, Perkin Elmer, Waltham, MA) was utilized to assess the structural stability of Nafarelin after processing. Fluorescence spectrum was recorded in the range of 300-500 nm.

In vitro Nafarelin release from CDS

For the release experiment the CDS was injected in 10 ml phosphate buffered saline (PBS) (pH= 7.4) at 37±1°C. During the experiment, the samples were shaken horizontally in an incubator shaker at 200 rpm. At predetermined time intervals, 0.5 ml of the sample was removed for identifying the amount of peptide released and the medium was replaced with the same quantity of fresh PBS to maintain the sink condition. In order to assess the effects of gel delivery system on the release behavior of free Nafarelin, the in vitro release study of free Nafarelin mixed with poloxamer-sodium alginate gel was also conducted using the same method.

Mathematical modeling

In order to evaluate the kinetic of Nafarelin release from CDS, the result of *in vitro* release studies was fitted to zero- order (Equation 3), first order (Equation 4), Korsmeyer's (Equation 5), Higuchi's (Equation 6) and Hixson- Crowell's (Equation 7) equations.

$$Q_t = Q_0 + K_0 t \quad \text{Equation 3}$$

$$\text{Log}Q_t = \log Q_0 - K_1 t / 2.303 \quad \text{Equation 4}$$

$$Q_t/Q_\infty = K_H t \quad \text{Equation 5}$$

$$Q_t/Q_\infty = Kt^n \quad \text{Equation 6}$$

$$(Q_t)^{1/3} = (Q_0)^{1/3} - K_s t \quad \text{Equation 7}$$

Where Q_t is the cumulative amount of drug released at time t, Q_0 is the initial amount of drug, Q_t/Q_0 is the fraction of drug released at time t, K_0, K_1, K_H and K_s are the release rate constants for zero, first, Higuchi's and Hixson- Crowell's model, K is the release rate constant and n is the release exponent.

Results

Design of experiments (DoE)

In order to determine the impact of independent variables on three dependent responses including entrapment efficiency, loading capacity, and size of NPs, Box-Behnken Response Surface Methodology was used. The results of 17 runs conducted by experimental design are

summarized in Table I. The quadratic model that mainly defines the relationship between independent and dependent parameters is introduced as below:

$$Y_{1,2,3} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_{12} + b_{22}X_{22} + b_{33}X_{32}$$

Y is the dependent factor referring to each factor level. X_1 to X_3 represent individual effect of independent factors, while X_1X_2, X_1X_3, and X_2X_3 show the interactions of variables on each other. b_0 is intercept; b_1, b_2 and b_3 are linear coefficients; b_{11}, b_{22} and b_{33} are quadratic coefficients. Consequences of the models and their coefficients were examined by Analysis of Variance (ANOVA). The size of NPs varied from 232 up to 657 nm. EE% has ranged from 38.53 to 88.59 and DL% varied from 0.64 to 6.03. The optimal formulation was set to present the maximum entrapment efficiency, maximum drug loading, and minimum particle size.

A quadratic model was fitted for EE% with significant p-value (<0.05) and insignificant lack of fit (p-value>0.05). The value of regression co-efficient (R^2) for this equation was 0.98 representing good correlation between the response and the selected variables. The effect of formulation parameters on EE% is given below:

$$EE\% = +43.88938 + 8.26301 * X_1 + 1.27009 \times 10^{-3} * X_2 + 97.81383 * X_3 - 3.18998 * X_1^2 - 57.00215 * X_3^2 + 5.04416 * X_1 * X_3 - 0.014420 * X_2 * X_3$$

DOE results indicate that PVA concentration (X_1), drug concentration (X_2), the interaction between PVA-polymer (X_1X_3) and drug-polymer concentration (X_2X_3) have momentous influence on the EE% of NPs (p-value<0.05). High EE% is achieved with high PVA concentration. EE% is inversely related to drug concentration.

Fig. 1a shows the effect of PVA and PHBV concentration on EE%. In both low and high PVA concentrations, the EE% was increased up to middle point and after that a descending trend was observed.

Fig. 1b indicates that similar to the effect of PVA-polymer concentration on EE% by increasing polymer concentration (regardless of drug concentration levels) the EE% was followed by an ascending trend and after that the EE% was slowly declined.

The following modified quadratic equation was fitted for loading capacity with p-value<0.05 and lack of fit>0.05. The value of regression co-efficient (R^2) for this model is 0.98.

$$DL\% = -2.49163 + 0.98753 * X_1 + 3.47368\times10^{-3} * X_2 + 12.30696 * X_3 - 1.12706\times10^{-6} * X_2^2 - 6.92105 * X_3^2 - 0.51169 * X_1 * X_3 - 8.43750\times10\text{-}4 * X_2 * X_3$$

These results indicate that PVA concentration (X_1) and polymer concentration (X_3) significantly influence loading capacity (p-value<0.05), whereas drug concentration alone has little impact (p-value>0.05). PVA concentration in interaction with polymer concentration (X_1X_3) and drug concentration in interaction with polymer concentration (X_2X_3) have significant impact on drug loading (p-value<0.05). Sharp increase in DL% was attained by an increase in polymer concentration level up to an intermediate point, howerve, after passing that point a descending trend was observed (Fig. 2a). Enhancement in PVA concentration has resulted in a sharp increase in DL%.

The quadratic function was significant for the particle size (p-value<0.05, lack of fit>0.05) with R^2 value of 0.98.

$$Size = +545.35676 - 136.22373 * X_1 - 0.21037 * X_2 - 461.19921 * X_3 + 37.42149 * X_1^2 + 7.1953 \times 10^{-5} * X_2^2 + 219.23469 * X_3^2 + 0/030909 * X_1 * X_2 + 63.76623 * X_1 * X_3 + 0.068437 * X_2 * X_3$$

The size of NPs has been shown to be affected by PVA concentration (X_1), drug concentration (X_2), PHBV concentration (X_3) and the interaction between these three parameters (p-value<0.05). As represented in Fig. 3, the minimum particle size was obtained when all the three variables had the lowest concentration level.

Optimization procedure

Based on the information obtained above, the optimized formulation consisted of 1.65% PVA, 835 μg/ml Nafarelin, and 0.78% PHBV. Design expert predicted 239.73 nm for particle size, 88.59% for EE, and 5.43% for DL. The results achieved from practical experiments were compared with the responses predicted by the software. In practice, NPs showed size of 248.6 nm, EE% of 85.84%, and DL% of 5.27% for the aforementioned responses. All the experimentally achieved values were within the confidence intervals (95%) of the predicted values; thus, the reliability and predictiveness of the regression was confirmed.

Nanoparticles size analysis

The results of DLS demonstrated that the average diameter of optimized formulation was 248.6 nm with PDI of 0.17 and zeta potential was -0.704 mV.

Morphological Characterization

The morphology of the optimized NPs was analyzed by SEM micrograph. As illustrated in Fig. 4, the optimized NPs exhibited spherical shape. No roughness or sharp edges were observed.

Differential scanning calorimetry (DSC)

The DSC thermal curves for NPs, PHBV, and Nafarelin are presented in Fig. 5. The thermograms showed endothermic peaks. For the NPs, the interaction energy was 101.48J/g (the temperature range from 97.29°C to 122.84°C) and 38.14J/g (the temperature range from 156.01°C to 173.14°C). The transition energy for PHBV and the pure Nafarelin were 68.24J/g (the temperature range from 167.06°C to 182.63°C) and 334J/g (the temperature range from 49.51°C to 132.05°C), respectively. The summary of thermal parameters is presented in Table 2.

In vitro Nafarelin release from optimized PHBV NPs

Fig. 6 displays *in vitro* release profile of free Nafarelin and Nafarelin loaded PHBV NPs in pH 7.4 at 37°C. Free Nafarelin shows 98.4% release after 12 hours which indicates the dialysis bag

had no retaining effect on drug release. The release pattern shows a burst release within 12 hours. 54.62% of Nafarelin from PHBV NPs was released between first and 7th days. Between day 7 to 13, a sustained release pattern was detected. After day 13, a second fast release occurred. In the last days of the study, the drug release profile reached the plateau stage. The release profile demonstrated that 90.21% of Nafarelin was released from NPs during 30 days.

Rheological properties

Fig. 7. shows elastic and viscous modulus versus temperature curves at a frequency value of 1 Hz for three formulations. Sol-gel transition temperature could be easily determined to be 41°C, 36°C and 32°C for 14% poloxamer 407, 14% poloxamer 407- 0.1% sodium alginate and 14% poloxamer 407- 0.3% sodium alginate, respectively. Thus, increasing in sodium alginate concentration from 0.1% to 0.3% led to decrease in the sol- gel transition temperature.

Scanning electron microscopy (SEM)

PHBV NPs integrity in poloxamer gel evaluated by SEM is displayed in Fig. 8. As it can be seen in Fig. 8, NPs kept their spherical shapes in gel system and there were no significant differences between sizes of NPs before and after loading in poloxamer gel.

Structural analysis of Nafarelin loaded CDS

The fluorescence emission intensity of pure Nafarelin (as a control) and Nafarelin loaded CDS exhibited similar absorption at 340 nm. Neither right shift nor left shift could be figured out in the two spectra which proves no signs of any structural changes during formulation.

In vitro Nafarelin release from CDS

Fig. 9 shows the cumulative release profile of Nafarelin from CDS in ratios of 2:1, 1:1, and 0.5:1. It was found that *in situ* gel system could sustain the drug release profile. The release pattern has no burst release. In case of 0.5:1 NPs/ *in situ* gel, the drug release was about 85.64% after 60 days. Nearly, 48% of Nafarelin was released to the medium from 1:1 ratio of NPs/ *in situ* gel after 60 days. For the 2:1 ratio of NPs/ *in situ* gel, significant lower drug release was detected. Approximately, around 25% of Nafarelin was released after 60 days. As it is shown in Fig.9, the release profile of free Nafarelin from poloxamer- sodium alginate gel represented an immediate release resulted in about 75.5% initial burst release within 12 hours. On the other hands, 91.5% of total Nafarelin released from poloxamer- sodium alginate gel in an uncontrolled manner within 48 hours.

Mathematical modeling

The Nafarelin CDS profile was analyzed using different kinetic models including zero- order, first order, Korsmeyer-Peppas, Higuchi and the Hixson- Crowell. The release kinetic parameters are shown in Table 3, where the correlation coefficient (R- square) is the release rate constant. It

was observed that the release data of Nafarelin nanoparticles followed the Higuchi model, with R^2 =0.89.

Discussion

In the current study, a unique prolonged formulation of Nafarelin based on PHBV NPs has been successfully prepared and statistically optimized by Box-Behnken design. The design of experiments is identified as a reliable method that assists to determine the impacts of different parameters on the output of the process (Baş 2007; Bahari Javan 2016). The results achieved in this study demonstrates that the quadratic model was the best fitted model for all dependent variables. Moreover, ANOVA provision was used for validation of the responses.

Effect of PVA concentration

The DOE results indicate that PVA concentration has significant impact on EE, size and DL of NPs.

An increase in PVA concentration level may lead to an escalation in the viscosity of external phase which prevents the diffusion of therapeutic agent from W_1 to the external phase which eventually leads to an increase in EE (Yang 2001; Mahboubian 2010). Ghasemian et al. determined that a higher PVA concentration level enhanced the EE of Sildenafil Citrate loaded PLGA NPs (Ghasemian 2013). In another study, PVA enhanced the drug EE by stabilizing the emulsion preparation of Fingolimod- loaded PHBV NPs (Rezaie Shirmard 2015).

The observations showed that increasing the amount of PVA may form a thin layer around the particles which led to an increase in the size of NPs (Guhagarkar 2009; Mahboubian

2010; Fiedot 2015). Additionally, it can be assumed that employing higher concentration of PVA decreases the mixing rate at the time of preparation which will then results in formation of larger NPs (Yanga 2001; Jayamanti 2014). Bahari Javan et al. demonstrated that PVA increases the size of Teriparatide- loaded PHBV/ PLGA NPs due to viscosity enhancement of PVA (Bahari Javan 2016).

Effect of polymer concentration

According to DOE results, polymer concentration has significant impact on all dependent responses.

An increase in polymer concentration led to an escalation in EE and DL up to the middle point, however, a descending trend was observed afterwards. Before the middle point, the higher polymer concentration level meant a faster polymer solidification at the surface of the internal aqueous phase. This restricts the drug diffusion and results in an increase in both EE and DL. Marquette et al. concluded that higher concentration of polymer led to a faster polymer precipitation at the surface of the internal phase reduced the drug diffusion into external phase in immunoglobulin G encapsulated in PLGA microspheres (Marquette 2014).

After the middle point, the different properties of polymer and therapeutic agent in hydrophilicity were responsible for the obtained results. The hydrophobic property of PHBV in comparison to hydrophilic nature of Nafarelin was considered as an obstacle for more entrapment of peptide within the polymer, therefore, by adjusting the polymer- drug ratio, the maximum EE and DL was perceived (Zhu 2009; Behera 2013). Bahari Javan et al. represented that the conflicting properties of PHBV and Teriparatide prevents hydrophilic drug from more entrapment into hydrophobic polymer (Bahari Javan 2016).

An increase in the size of NPs as a result of an escalated level of polymer concentration after middle point, could be explained by the increase in the polymer solution viscosity, which affected the shearing capacity of homogenization and eventually resulted in creation of larger NPs (Behera 2013; Elwerfalli 2015; Gu 2015). Furthermore, polymer solidification in high concentration of PHBV may enhance the aggregation of NPs, resulting in particle growth. Cun et al. concluded that the increase in polymer concentration enhances the size of siRNA encapsulated in PLGA NPs (Cun 2011).

The impact of drug concentration

Further to DOE results, drug concentration has significant effects on size of NPs.

The size of NPs increased with an increase in the drug concentration. This could be due to the conflicting properties of polymer and drug in hydrophilicity. By increasing the concentration of hydrophilic drug, the hydrophobic polymer may prevent the drug from more entrapment, therefore, the additional drug aggregate on the surface of NPs may result in an increase in NPs size (Noori 2014).

The optimized formulation showed a non-significant difference from the predicted values, demonstrating an excellent prediction by this method. High DL% and EE% reduces the quantity of the drug needed which makes it cost effective and lowers the adverse effects.

DLS and SEM studies of the optimized formulation revealed that NPs were uniform in size and spherical in shape. The particle size analysis indicated by SEM displayed smaller size for optimized NPs than the results obtained from size distribution test. Since the laser scattering method measured the hydrodynamic size of the NPs, the size of NPs is exaggerated.

Furthermore, the DLS method can be influenced by dust particles or/and aggregations and therefore represents the larger particle size (Bootz 2004). The PDI of 0.174 is an indicator of uniformity of the size distribution. The zeta potential shows the stability of NPs in a specific solution. A zeta potential of Nafarelin loaded PHBV NPs was -0.704 mV. This could be due to the presence of PVA during formulation. PVA also decreases the repulsion between the particles (Want 2014).

Presence of two endothermic peaks in DSC thermogram of NPs proved the physical mixture of both components. The endothermic peak at 177°C was corresponded to pure PHBV (Mendes 2012; Saadat 2015; Wei 2015). When drug molecules surrounded with polymers, their phase transition point generally shift to a different temperature. This slight change in drug and polymer thermograms could be the result of morphological changes during the formulation (Derakhshandeh 2010). Nafarelin transition peak was unaffected by loading in the PHBV NPs which proves the maintenance of its original state. These results are in agreement with a previous study in which the DSC thermogram of Zidovudine and Zidovudine loaded PLGA NPs was studied. Christoper et al. concluded that the Zidovudine loaded PLGA NPs shows two endothermic peak to be nominated for the polymer and drug that shows the entrapment of drug in polymer without any interaction. Also the slight change in Zidovudine thermogram was derived from the morphological changes after formulation (Christoper 2014).

The amount of free Nafarelin detected after 12 hours indicates that the dialysis bag has no retaining effect on drug release. The initial burst release observed after 12 hours might be due to the Nafarelin remained unentrapped on the surface of PHBV. After the initial sudden release, the slow degradation of PHBV causes a sustained release pattern up to day 13. Slight drug diffusion from PHBV NPs might be due to the hydrophobic polymer matrix that causes PBS to penetrate

into the polymer matrix slowly (Islam 2013). Swift release after day 13, was attributed to formation of intramolecular linkages in the matrix of polymers and sudden polymer degradation. Niwa et al, reported that only 60% of Nafarelin was released from PLGA NPs after 20 hours (Niwa 1994). The *in vitro* result presented here, can be compared to the result obtained by previous study which stated that PHBV NPs could provide a well-ordered release of Nafarelin overtime compared to Nafarelin loaded PLGA NPs.

After optimization of PHBV NPs, they were loaded in poloxamer 407/ sodium alginate *in situ* gel to prepare the CDS in order to diminish the burst release. The slight shrinkage in the structure of NPs after loading in gel can be attributed to the outward diffusion of Nafarelin from the inner pores of the NPs to the surface part of NPs (Öchsner 2013). Previous works mainly introduced solvent evaporation as the most important cause of shrinkage (Pain 2000; Kudo 2001). Ayal et al. assumed that solvent evaporation after exposure to SEM could be the dominant reason of the shrinkage. Using a more volatile solvent or heating for longer period of time can reduce the remained solvent in the formulation, which cause less solvent evaporation when it is exposed to SEM radiation and thus the shrinkage will be lessened (Ayal 2009). In the current study, since the SEM images demonstrated that dimensional changes did not occur to the NPs after loading in gel, the shrinkage could be considered negligible.

In a study carried out by Ramyadevi et al., Acyclovir loaded polyvinyl pyrrolidon-Eudragit RSPO hybrid polymeric NPs were entrapped in 15% pluronic F-127 gel to improve permeability and bioavailability through vaginal membrane (Ramyadevi 2016). Upadhayay et al. prepared a CDS based on chitosan/ sodium tripolyphosphate (TPP) NPs loaded in carbopol 934 *in situ* gel to improve erosion time by the tear fluid and have a longer residence time of the drug (Upadhyay 2016). Teng et al. loaded ethyl cellulose NPs in poloxamer 188/ poloxamer 407 and

carbopol 940 gel to improve the bioavailability of Loratadine in comparison with the oral solution (Teng date unknown).

In order to investigate the effects of sodium alginate as a mucoadhesive polymer on the rheological properties of poloxamer solution as a thermosensitive polymer, rheological study was performed. When the formulation is in liquid consistency, viscous modulus is higher than elastic modulus (G" > G'). When the temperature is increased, both viscous modulus and elastic modulus escalate. However, the increase of elastic modulus is more than storage modulus which results in elastic modulus to be higher than storage modulus after gelation (G' > G"). Based on our studies, sodium alginate changes the gelation temperature. Increasing the concentration of sodium alginate causes reduction in sol-gel transition temperature. The result is also in agreement with a previous study in which adding hyaluronic acid (HA) to poloxamer 407 and increasing the concentration of HA both decrease the sol-gel transition temperature (Mayol 2008). The gelation process of all three formulations was very quick which could be advantageous in preventing instant gel leakage after administration. The sol-gel transition temperature should be near body temperature (≈37°C) and due to results, 14% poloxamer 407-0.1% sodium alginate could be the most suitable formulation.

Fluorescence spectroscopy was utilized to study protein absorption on aromatic residues. In fluorescence spectroscopy, a beam of light (usually ultraviolet light), excites the electrons in three types of amino acids (tryptophan, tyrosine and phenylalanine) and causes them to emit visible light. Tryptophan fluorescence was strongly influenced by proximity of peptide bonds. Any conversion of amino acid side chains or backbone can affect the tryptophan emission spectrum (Lakowicz 2006). In the present study the overlapping of emission bonds proved the structural stability of Nafarelin loaded CDS.

Based on the results achieved in this study, an increase in the NPs: *in situ* gel ratio did not cause an enhancement in drug release from CDS. Two reasons might be involved for this phenomenon. Firstly, an increasing in NPs: *in situ* gel ratio might unfavorably enhance the rigidity and strength of the CDS and thus it restricts the facile diffusion of aqueous medium to the matrix of CDS for the further release. In other words, increasing in NPs: *in situ* gel ratio was considered as a vain attempt in increasing the percentage of release due to undesirable increase in the rigidity of the CDS and remarkable decrease in diffusion of aqueous medium to the CDS. Secondly, it is hypothesized that OH groups in poloxamer- alginate gel might be adsorbed by the surface of PHBV NPs and caused undesirable interactions between NPs and gel, thereby resulting in retained effect. Hence, the constructed CDS was not able to release all the amounts of Nafarelin. Similar to the present work, in a study conducted by Moebus and colleagues density of the polymer network was introduced as an effective parameter in taking up aqueous medium to the matrix of the drug delivery system (Moebus 2009). Further to the previous studies, van der waals and chemical interactions were extensively introduced as the reasons of release reduction for different therapeutic agents (Ukmar 2011a; Ukmar 2011b; Bahari Javan 2017). As a consequence, ratio 0.5:1 of NPs: *in situ* gel was the most appropriate formulation which resulted in 85% release over 60 days, while drug release from the two others ratios of NPs/ *in situ* gel was less than 50% in 60 days. The free Nafarelin release profile from poloxamer-sodium alginate gel exhibits a swift burst release, which was significantly higher than both NPs and CDS. Relatively large amounts of Nafarelin was adsorbed to the surface of the gel and released abruptly. Since 91.5% of Nafarelin released from poloxamer- sodium alginate gel in an uncontrolled manner within a very short time (48 hours), the *in situ* gel could not be a suitable drug delivery system to sustain and prolong the drug release. These results are in agreement with

a previous study, in which Acyclovir loaded poloxmer 407- carrageenan gel showed about 95% burst release within 10 hours (Liu 2009). In another study conducted by Dangre et al. 85.82% of total Chloramphenicol was released from 18% poloxamer 407 gel within 8 hours (Dangre 2013). The results achieved in this study, indicate that the appropriate controlled release pattern is not fully achieved by using *in situ* gel system alone and entrapment the NPs in *in situ* gel system has a synergistic effect on sustaining the drug release. The initial burst release observed in the release profile of Nafarelin from PHBV NPs vanished by loading Nafarelin- PHBV NPs in *in situ* gel. Teng et al. studied the release profile of Loratadine from ethyl cellulose NPs and CDS based on poloxamer 188/ poloxamer 407 and carbopol 940 *in situ* gel. In this study Loratadine release from CDS was 88.1% in 24 hours while the drug release from optimized formulation of NPs was 85.74% in 8 hours (Teng date unknown). In another study, Liu et al, shows the Acyclovir release from poloxamer 407 *in situ* gel with different content of carrageenan slows the release of Acyclovir (Liu 2009). Also in the cited study, the influence of Acyclovir concentration on kinetic of release from *in situ* gel was observed and the conclusion (as proven by the current study) showed that increase in drug content decreases the drug release from *in situ* gel.

Conclusion

This study demonstrates that the combination delivery system consisting of sodium alginate/ poloxamer 407 *in situ* gel loaded with PHBV NPs can be a promising candidate for prolonged formulation of Nafarelin. After preparation of PHBV NPs by using double emulsion solvent evaporation technique, the size distribution, morphology, structural analysis, thermal analysis and *in vitro* release were evaluated. In order to optimize the EE%, DL% and the particle size, the experimental design was used. The PVA concentration was recognized as the most critical

factor. The *in vitro* study suggested that CDS could sustain the release of Nafarelin for a period of 60 days. Based on these findings, we assume that the combined system based on PHBV NPs loaded in poloxamer 407 *in situ* gel may be an ideal system for preparation of a controlled-release formulation of Nafarelin. This unique strategy is worth further *in vivo* studies for developing an injectable form of Nafarelin.

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Legends to figures

Fig. 1. Three dimensional plot showing the effect of (a) polymer and PVA concentration and (b) polymer and drug concentration on EE%.

Fig. 2. Three dimensional plot showing the effect of polymer concentratio and PVA concentration on DL%.

Fig. 3. Three dimensional plot showing the effect of (a) drug and PVA concentration, (b) polymer and PVA concentration, and (c) polymer and drug concentration on particle size.

Fig. 4. SEM image of optimized Nafarelin loaded PHBV NPs.

Fig. 5. DSC thermograms of NPs, PHBV and Nafarelin.

Fig. 6. *In vitro* release profile of Nafarelin from optimized NPs, n=3, data are shown as mean± SD.

Fig. 7. Change of elastic (G') and viscous modulus (G") versus temperature for three different formulations.

Fig. 8. SEM image of optimized Nafarelin- PHBV NPs after loading in *in situ* gel system.

Fig. 9. Release profile of free Nafarelin in poloxamer- sodium alginate gel and release profile of Nafarelin from different ratios of PHBV NPs loaded 14% poloxamer 407- 0.1% sodium alginate at 37°C, n=3, data are shown as mean± SD.

Table Captions

Table 1: Independent and dependent variable from DOE.

Table 2. Thermal parameters of NPs, PHBV, and Nafarelin obtained from DSC analyses.

Table 3. R square of Nafarelin CDS release kinetics by fitting in different mathematical models.

Std	Run	PVA[a] Conc (%)	Drug Conc (µg/ml)	Polymer Conc (%)	Size (nm)	EE[b] (%)	DL[c] (%)
8	1	3.00	1200.00	1.50	657.00	57.57	1.80
15	2	1.63	1200.00	0.80	300.00	81.83	5.30
12	3	1.63	2000.00	1.50	534.00	38.53	0.64
4	4	3.00	2000.00	0.80	569.00	73.40	6.03
7	5	0.25	1200.00	1.50	322.00	39.63	1.40
6	6	3.00	1200.00	0.10	420.00	52.97	3.87
9	7	1.63	400.00	0.10	358.00	58.83	1.52
2	8	3.00	400.00	0.80	365.00	86.30	5.39
3	9	0.25	2000.00	0.80	309.00	69.48	4.15
10	10	1.63	2000.00	0.10	360.70	56.56	2.21
14	11	1.63	1200.00	0.80	236.00	81.83	5.86
13	12	1.63	1200.00	0.80	245.00	88.59	5.40
17	13	1.63	1200.00	0.80	258.00	87.50	5.70
16	14	1.63	1200.00	0.80	232.00	85.62	6.00
5	15	0.25	1200.00	0.10	330.50	54.45	1.50
11	16	1.63	400.00	1.50	378.00	73.10	1.84
1	17	0.25	400.00	0.80	241.00	85.44	3.68

a: Poly Vinyl Alcohol

b: Entrapment Efficiency

c: Drug Loading

Sample	ΔH (J/g)	T_o [a] (°C)	T_p [b] (°C)	T_c [c] (°C)	T [d] (°C)	Peak Width (°C)
NPs	101.48	97.29	109.83	122.84	25.55	14.87
	38.14	156.01	166.33	173.14	17.13	10.20
PHBV	68.24	167.06	177.24	182.63	15.57	10.83
Nafarelin	334.00	49.51	98.17	132.05	82.54	50.52

a: onset temperature

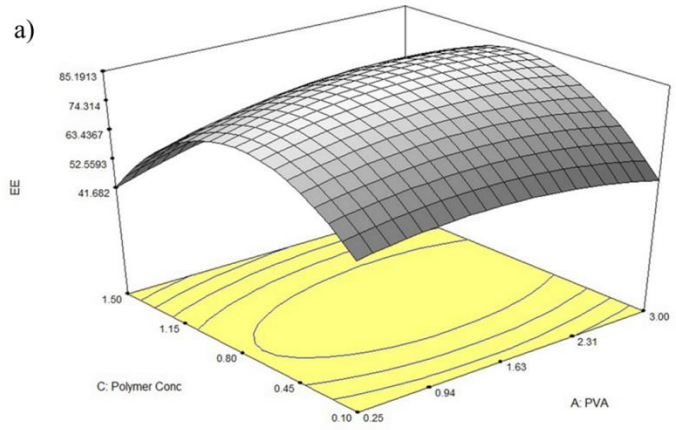
b: peak temperature

c: conclusion temperature

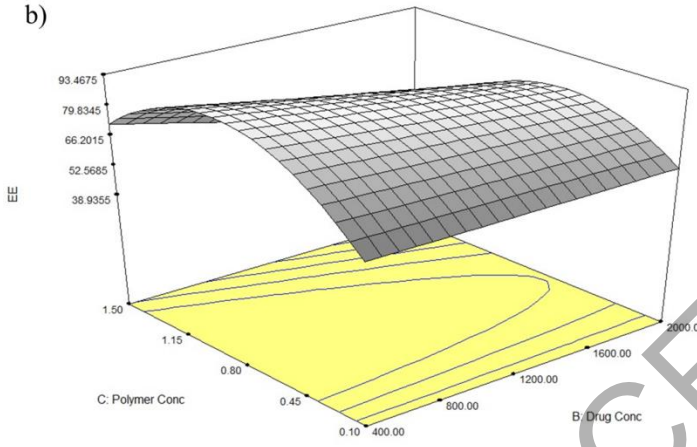
d: temperature range

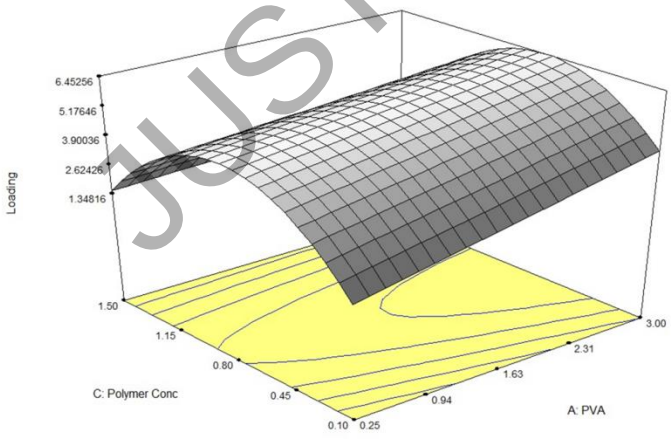
Fitted theoretical models					
	Zero-order	First-order	Korsmeyer-Peppas	Higuchi	Hixson-Crowell
R^2	0.684	0.388	0.562	0.893	0.799

a)

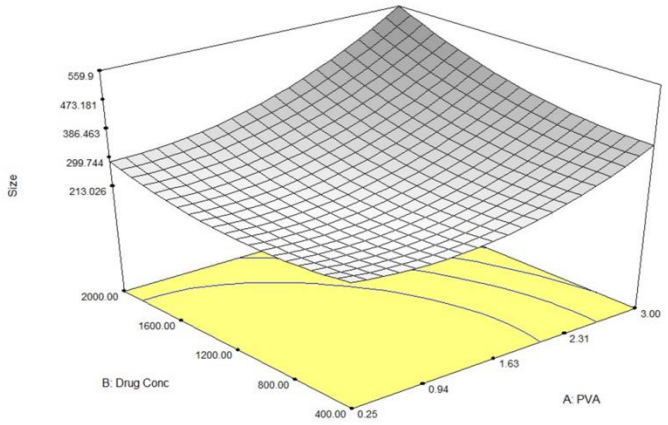


b)

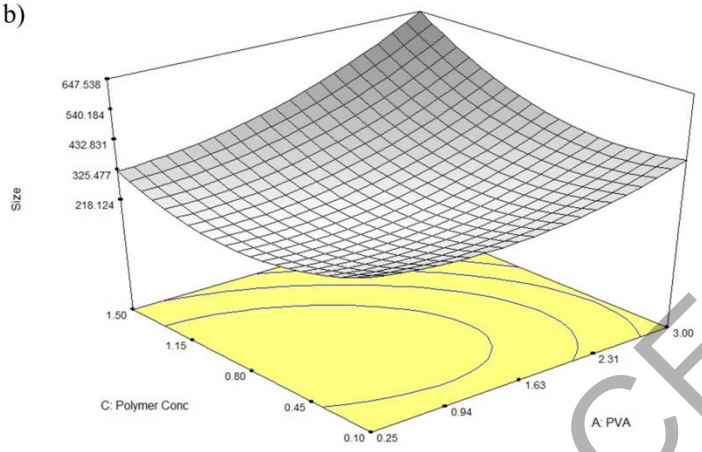




a)



b)



c)