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Research paper

Design of experiments approach for the development of a validated method to determine the exenatide content in poly (lactide-co-glycolide) microspheres

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ABSTRACT

Due to the lack of pharmacopeia guidelines for injectable microspheres based on poly (D, L-lactide-co-glycolide) (PLGA), an internal method validation is a critical prerequisite for quality assurance. One of the essential issues of developing peptide-based drugs loaded PLGA microspheres is the precise determination of the amount of peptide drug entrapped in the microspheres. The aim of this study is the development and optimization of a method for measuring the drug content loading of PLGA microspheres using exenatide as a model peptide drug. Exenatide-loaded PLGA microspheres were prepared by a double emulsion solvent evaporation method. The extraction method to determine exenatide content in microspheres was optimized using Design of Experiments (DoE) approach. After the initial screening of six factors, using Fractional Factorial design (FFD), four of them, including type of organic solvent, buffer/organic solvent ratio (v/v), shaking time and pH, exhibited significant effects on the response, namely the exenatide loading, and a Box-Behnken design (BBD) was subsequently applied to obtain its optimum level. The optimum level for organic solvent volume, buffer/organic solvent ratio, shaking time, and pH were 4 ml, 1, 5.6 hrs, and pH 6, respectively. The exenatide content in microspheres under these conditions was 6.4 ± 0.0 (%w/w), whereas a value of 6.1% was predicted by the derived equation. This excellent agreement between the actual and the predicted value demonstrates that the fitted model can thus be used to determine the exenatide content.

1. Introduction

In recent years, the development of prolonged-release formulations such as microspheres, implants, and liposomes has received tremendous attention. These formulations are developed to the improve the therapeutic efficacy of drugs, and also benefit patient compliance. Particularly polymeric microspheres have been widely used for subcutaneous and intramuscular injection to control the release of loaded low molecular weight drugs as well as biotherapeutics, such as proteins and peptides [1–4]. Due to the biodegradability and biocompatibility of poly (D, L-lactide-co-glycolide) (PLGA) [5,6], many studies have focused on microspheres based on these polymers for the development of controlled release systems for pharmaceutically active peptides and proteins [7,8]. In the last two decades, a number PLGA-based peptide formulations has been approved by FDA, including Lupron Depot®/Lupaneta Pack™ (Leuprolide), Decapeptyl®/Trelstar®/Triptodur Kit® (Triptorelin), Zoladex® (Goserelin), Sandostatin® LAR Depot (Octreotide), Signifor® LAR (Pasireotide), Bydureon® (Exenatide) [7,9,10].

Like other drug formulations, various characterization methods are needed to ensure the pharmaceutical quality of microsphere products. Different physicochemical properties of PLGA microspheres such as morphology, particle size (distribution) and zeta potential, drug

Abbreviations: PVA, Polyvinyl alcohol; PLGA, Poly (D, L-lactic-co-glycolic acid); DoE, Design of experiments; FFD, Fractional Factorial design; BBD, Box-Behnken design; ACN, Acetonitrile; DMSO, Dimethyl sulfoxide; RSM, Response surface methodology.

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encapsulation efficiency, drug content and drug release (*in vitro* and *in vivo*), residual organic solvent content, polymer molecular weight, glass transition temperature (T_g), and moisture content are usually established [11–14]. Due to the lack of pharmacopeia methods for injectable microspheres, an internal method validation is a critical prerequisite for quality assurance [15].

Analytical characterization of peptide drugs loaded in PLGA microspheres is challenging due to their complex structure. One of the critical issues in developing peptide/PLGA microsphere formulations is the precise determination of the drug content, defined as the weight fraction of drug in the microspheres [16]. The drug content can be determined using an indirect method, meaning that quantification of the amount of drug encapsulated in the microspheres is calculated by subtracting the amount of drug which is not incorporated from the total amount in the feed. Alternatively, the drug content is determined by a direct method by extraction of the bioactive from microspheres. The latter method is necessary for evaluating the quality of marketed drug and stability studies [17]. Accurate determination of the drug content is required for studying the effects of microencapsulation techniques and process variables on the encapsulation and loading efficiency as well as dosing the targeted amount of peptide in vials for *in vivo* studies, determination of the *in vitro* release kinetics, and for quality control of the final product during stability studies [18].

Various direct methods have been published to determine the amount of peptide entrapped in PLGA microspheres. One of these approaches is the hydrolysis of microspheres under acid or basic conditions followed by amino acid or peptide analyses [19]. Importantly, this method may be associated with peptide degradation due to hydrolysis of amide bonds. Another direct method concerns dissolution of the microspheres in a suitable solvent such as dichloromethane followed by extraction of the peptide with an aqueous medium [20,21]. It is obvious that incomplete extraction of the peptide from the organic phase to the aqueous phase leads to an underestimating of the peptide amount in the microspheres [22]. The most commonly used direct method is based on the dissolution of the polymer and peptide in a water-miscible organic solvent such as acetonitrile (ACN), acetone, and dimethyl sulfoxide (DMSO). After obtaining a dispersion (due to the presence of particles of insoluble peptide), an aqueous buffer solution is added, which results in precipitation of PLGA and dissolution of the peptide [23]. Using this method, it cannot be avoided that the peptide co-precipitates with PLGA and/or stick to the precipitated polymer, which as a consequence results in an underestimation of the loaded amount.

Exenatide is a polypeptide of 39 amino acids, with a molecular weight of 4186 Da and an isoelectric point of pH 4.86 [24].This drug is a glucagon-like peptide (GLP-1) agonist approved for treating type 2 diabetes. Low patient compliance, short half-life, and kidney toxicity are the most important challenges posed by multiple daily injections [25]. Exenatide was first registered by Byetta® in 2009 and is administered twice daily as a subcutaneous injection in the thigh, upper arm, or abdomen. More recently, Bydureon® was introduced, which is a once-weekly injection product based on exenatide-loaded PLGA microsphere, approved by FDA in 2014 for treatment of adults with type-2 diabetes [24,26,27].

In the literature, different methods were reported for the determination of the exenatide content of this PLGA microsphere formulation. For example, Wang et al. measured the exenatide content by dissolving 10 mg of PLGA microspheres in 5 ml of ACN, followed by the addition of the same volume of acetate buffer of pH 4.5. The mixture was subsequently centrifuged, and the peptide in the supernatant was quantified by HPLC analysis [28]. In another study, Qi et al. determined the exenatide content of PLGA microspheres by dissolving the microspheres (5 mg) in a mixture of ACN and 0.01 M HCl 15/85 (v/v). However, the dissolution of PLGA in this mixture is questionable [29]. In a study of Tinghui Li et al. the peptide content of marketed exenatide microspheres (Bydureon®), was determined using a sophisticated method comprise five extraction steps with acetone followed by drying. The exenatide content was determined by UPLC and N-content analysis. It turned out that the exenatide content of Bydureon® was 4.47 ± 0.04% (w/w) and 4.89 ± 0.02% (w/w) by UPLC and N analysis, respectively. These reported values are slightly different from each other, however, are close to the 5% loading as claimed on the package insert [30].

The discussed examples highlight the problem that despite the high interest in peptide formulations based on PLGA, a convenient method for quantifying the drug content is still lacking. In other words, insufficient attention has been paid to the development, optimization, and validation of appropriate drug content assay methods for PLGA/peptide formulations.

Design of experiments (DoE) is a powerful and systematized methodology for optimization and validation of processes and products by utilizing the principles of statistics [31]. DoE gives important insights how the relationship between different factors and selected response(s) by testing a minimum number of experiments to yield maximal responses, thus saving time and simultaneously reducing costs [32]. In contrast to traditional one factor at a time (OFAT) approach, the DoE methodology considers interactions between factors, improves the accuracy and reduction of bias errors, and thus provides more information about the studied system [32–34]. The Fractional Factorial design (FFD) can be applied to identify the most critical variables, which makes the study relatively simple and well-manageable [35]. After selecting the most important variables influencing response(s) in the screening study, Response Surface Methodology (RSM) is normally applied to optimize the factors and understand the relationship between the main factors, response(s) and their interactions in an efficient and suitable model [36].

This study aims to use the DoE methodology to develop and optimize a water-miscible organic solvent method for determination of the drug content of PLGA microspheres using exenatide as a model peptide. Process parameters, including the type of organic solvent, buffer/organic solvent ratio, pH of the aqueous phase, and effect salts, were studied to identify a method for determining the amount of exenatide content of PLGA microspheres with high reproducibility and efficiency, which not only for exenatide is also applicable for both laboratory and industrial uses.

2. Materials and methods

2.1. Materials

PLGA 50:50 (acid terminated 5004A with lactide/glycolide molar ratio 50:50, and inherent viscosity of 0.4 dl/g) was purchased from Corbion, the Netherlands. Bydureon® (Lot. number LC0144) was purchased from a local pharmacy in Germany and shipped in a well-conditioned cold pack. Exenatide (purity 98.5%) was kindly provided by Parsian Pharmaceutical Co (Iran). Polyvinyl alcohol (PVA; MW 30,000–70,000; 88% hydrolyzed) was obtained from Sigma Chemical Co. (St. Louis, MO). ACN and trifluoroacetic acid (both of HPLC grade) were purchased from Sigma Chemical Co. (St. Louis, MO). NaCl, NaH_2PO_4, $MgCl_2$, and DMSO were obtained from Merck (Darmstadt, Germany), and all other chemical reagents used for analytical methods were of at least analytical grade.

2.2. Preparation of the exenatide microspheres

A double emulsion (w/o/w) solvent evaporation technique, as described by Shirangi et al., was used to prepare exenatide-loaded microspheres [37]. Briefly, 100 μl of an exenatide solution in Mili Q water (300 mg/ml) was emulsified with 1000 μl PLGA solution in dichloromethane (300 mg, 18.4% w/w). The mixture was homogenized at the highest speed (30,000 rpm) for 30 s using a MICCRA homogenizer (MICCRA D-1, Germany) to obtain a w/o emulsion. Then, 2000 μl of PVA solution (1% w/w in 30 mM acetate buffer, pH 4.5) was added, and the mixture was vortexed for 45 s at 30,000 rpm. Next, the resulting w/

Table 1
Independent Variables used in screening by the FFD.

Independent variable	Unit	Symbol	Low level (–1)	Medium level (0)	High level (+1)
Solvent type	–	X_1	ACN	–	DMSO
Buffer/organic solvent ratio	–	X_2	1	2	3
$MgCl_2$	mM	X_3	0	125	250
Tween 80 (%w/v)	g/L	X_4	0	0.25	0.5
Shaking time	hrs	X_5	2	5	8
pH	-log H+	X_6	2.5	4.5	6

o/w emulsion was transferred into an aqueous solution (7.5 ml PVA 0.5% w/w in 30 mM buffer acetate pH 4.5) and stirred for 2 hrs at ambient temperature to evaporate DCM. The hardened microspheres were collected by centrifugation (HETTICH EBA 20, Canada) at 5,000 g for 3 min, followed by washing three times with 50 ml of RO water. The resulting microspheres were lyophilized using a freeze-dryer (Christ, Switzerland). Main drying phase took place at −50 °C and 0.005 mbar for 24 hrs followed by a final drying which took place at 1 mbar and the temperature gradually elevated to 20 °C over a period of 2hrs. The freeze-dried microspheres were subsequently stored at 20 °C.

2.3. Optimization of the method of exenatide content assay for PLGA microspheres using DoE

The exenatide content of the PLGA microspheres prepared as described in section 2.2. was determined by dissolving accurately weighed amounts of microspheres of ~10 mg in 2–4 ml of organic solvent (ACN or DMSO) with gentle shaking (100 rpm, 37° C) (Memmert, Germany). Next, 2–12 ml of acetate buffer solutions (100 mM, pH 2.5, 4.5 or 6.0) was added to precipitate PLGA. Next, the obtained mixture was centrifuged at 5,000 g for 3 min. The amount of exenatide in the supernatant was quantified using HPLC analysis (Agilent, 1260 infinity) with a reverse phase column BEH x-bridge Waters C18 (5 μm, 4.6 × 150 mm). In detail, a linear gradient of mobile phase A (95% H_2O, 5% ACN and 0.1% (v/v) TFA) and mobile phase B (100% ACN and 0.1% (v/v) TFA) was used. The eluent linearity changed from 25 to 90% A over 12 min at a flow rate of 0.8 ml/min. Exenatide standards (7.8–250 μg/ml, injection volume 20 μl) were used for calibration, and detection was done at 210 and 280 nm. The drug content (%) of microspheres was calculated as follows [28]:

$$\text{Drug Content}\ (\%) = \frac{\text{weight of exenatide in the microspheres}}{\text{weight of the drug} - \text{loaded microspheres}} \times 100 \qquad (1)$$

2.3.1. Experimental design and statistical analysis

To identify the critical independent variables influencing the method of determining exenatide content for PLGA microspheres, a screening design was performed. The next step in the process was the use of a Box-Behnken design (BBD) to obtain a response surface to identify the best extraction procedure for determination of the drug content.

2.3.1.1. Screening design. The factors affecting the extraction of exenatide from PLGA microspheres were chosen as follows: To determine the amount of loaded exenatide, the microspheres were dissolved in a water-miscible organic solvent. After addition an excess of buffer solution, PLGA precipitates. Using this procedure, exenatide may co-precipitate with the polymer resulting in a decrease of the recovery of the peptide. DMSO and ACN were chosen as the organic solvents because they solubilize both exenatide and PLGA to yield a homogeneous phase [38]. The pH of the buffer may affect both the solubility and stability of the peptide [38]. In addition, the shaking time can play an important role for the extraction of exenatide from the microspheres. To disrupt the electrostatic interaction between the carboxylate groups of the PLGA chains and the amine groups of exenatide and thus to prevent the co-precipitating of the peptide with polymer, $MgCl_2$ was added [39]. Furthermore, tween 80 was added to prevent adsorption of exenatide onto the wall of the container and simultaneously improve its stability.

A 2-level FFD (2_{IV}^{6-2}) with six central points was utilized to identify the important variables that potentially have significant effects on the measured exenatide content of the microspheres. In detail, solvent type (X_1), buffer/organic solvent ratio (X_2), $MgCl_2$ concentration (X_3), tween 80 concentration (X_4), shaking time (X_5), and pH (X_6) were selected as independent variables (Table 1). The measured exenatide content was the dependent variable.

As shown in Table 1, the identified independent variables were varied at three different levels, which are coded as low (−1), medium (0), and high (+1). The Design-Expert software® (9.0.4.1, Stat-Ease Inc., Minneapolis, USA) was applied to evaluate six factors in twenty-two experiments.

Table 2
Independent Variables used in the BBD.

Independent variable	Unit	Symbol	Low level (–1)	Center point (0)	High level (+1)
ACN	*ml*	X_1	2	3	4
Buffer/ organic solvent ratio	–	X_2	1	2	3
Shaking time	*hrs*	X_3	2	5	8
pH	–	X_4	2.5	4.5	6

2.3.1.2. Optimization design. After identifying the most important factors affecting the response, the optimum level of each factor was distinguished using an RSM. Optimization of the level of variables affecting the measured exenatide content of the microspheres was carried out using a BBD.

Factors that were found significant in FFD, including ACN volume (X_1), buffer/organic solvent volume ratio (X_2), shaking time (X_3), and pH (X_4) were defined at three levels, which are coded as low (-1), medium (0), and high (+1) (Table 2). Based on Design-Expert software, twenty-nine experiments, including twenty-three factorial points, with six replicates at center points to estimate the pure error and the sum of squares, were performed.

Design-Expert was utilized for statistical analysis of the data by employing multiple regression modeling and plotting the response surface graphs. In addition, the significance of the effect of the independent factors on the response was investigated by analysis of variance (ANOVA) through Fisher's test and a value of $p < 0.05$ was considered significant.

The mathematical relevance between response (Y) and independent variables (Xi) was modeled using the following quadratic polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \beta_{44} {X_4}^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \qquad (2)$$

Where Y is the predicted response, (X_1, X_2, X_3, X_4) represent the independent variables, β_0 is the intercept, (β_1, β_2, β_3, β_4) are the linear coefficients, (β_{11}, β_{22}, β_{33}, β_{44}) are the quadratic coefficient of the independent variables, and (β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34}) represent the interaction coefficients between the four defined independent factors, respectively. Using this equation, it is possible to assess linear, quadratic, and interactive effects of the independent variables on the response. The quality of the fit of the second-order polynomial equation was evaluated using the multiple correlation coefficient (R^2), adjusted R^2, and predicted R^2 [40].

Table 3
Analysis of variance for FFD.

Source	Sum of squares	df	Mean square	F-value	*p*-value	Effects	%Contribution
Model	15.22	13	1.17	177.21	< 0.0001	–	–
X_1	2.98	1	2.98	450.77	< 0.0001	−0.86	19.5
X_2	1.43	1	1.43	216.80	< 0.0001	−0.60	9.38
X_3	0.10	1	0.10	15.30	0.0079	0.16	0.66
X_4	0.18	1	0.18	27.20	0.0020	0.21	1.18
X_5	1.06	1	1.06	160.21	< 0.0001	0.51	6.93
X_6	3.33	1	3.33	503.41	< 0.0001	0.91	21.77
X_1X_2	0.58	1	0.58	87.51	< 0.0001	–	–
X_1X_4	0.67	1	0.67	100.63	< 0.0001	–	–
X_1X_2	2.62	1	2.62	396.36	< 0.0001	–	–
X_1X_6	0.32	1	75.87	48.05	0.0004	–	–
Lack of Fit	0.014	2	6.886E-003	1.06	0.4260	–	–

Table 4
Analysis of variance of the fitted model based on Box-Behnken design for the measured exenatide content of the PLGA microspheres.

Source	Sum of Squares	df	Mean Square	F-Value	p-value	
Model	13.18	5	2.64	20.33	< 0.0001	Significant
X_1	1.25	1	1.25	9.67	0.0051	Significant
X_2	1.74	1	1.74	13.39	0.0014	Significant
X_4	6.25	1	6.25	48.21	< 0.0001	Significant
X_2^2	1.67	1	1.67	12.86	0.0016	Significant
X_4^2	1.62	1	1.62	12.47	0.0019	Significant
Residual	2.85	22	0.13			
Lack of Fit	2.80	19	0.15	8.78	0.05	Not Significant
Pure Error	0.050	3	0.017			
Cor. Total	16.03	27				
R^2	0.82					
Adj. R^2	0.78					

3. Results and discussion

3.1. Fractional Factorial design for screening important factors

In the first step of optimization, the effect of six factors on the measured exenatide content of the PLGA microspheres was evaluated using an FFD. The design matrix and the results are presented in Table SI 1. As shown in Table 3, statistical analysis using a student *t*-test demonstrated that the model p-value was <0.05. In addition, all independent variables tested had a statistically significant effect (p-value < 0.05) on the determined exenatide content. pH, solvent type, buffer: organic solvent ratio, and shaking time were the primary factors with the highest normalized effect on the measured exenatide content. However, as presented in Table 3, the effects of $MgCl_2$ (X_4) and tween80 (X_5) were 0.66% and 1.18%, respectively. Although these independent variables had significant effects (p-value < 0.05), due to their relatively small effect compared to the other selected independent variables, these two factors were excluded in the optimization stage and thus the effects of pH, solvent type, buffer: organic solvent ratio, and shaking time on the amount extracted of drug were further investigated.

3.2. Box-Behnken design (BBD) and for response surface methodology for optimization

After the screening design as discussed in Section 3.3, in order to select the optimum level of each factor using the Design-Expert software, a BBD was implemented to design an experimental matrix, to perform statistical analysis and to develop a second order polynomial model. The design matrix and the results obtained are shown in Table SI 2. The exenatide content ranged from 2.7 to 6.5% (w/w). The best model to fit data was obtained by analysis of variance by calculating F-value. The model was fitted successfully ($p < 0.05$), and the ACN volume (X_1), buffer/organic solvent ratio (X_2), and pH (X_4) were identified as significant variables (Table 4).

The calculated mismatch of R^2, adjusted R^2, and predicted R^2 were 0.82, 0.78, and 0.67, respectively. The difference between the predicted and fitted R^2 was <0.22, indicating reasonable agreement between predicted and experimental results, and the following equation was fitted to the model.

$$Y = +4.64 + 0.3232X_1 - 0.3803X_2 + 0.7218X_4 + 0.5001X_{22}^2 - 0.4925X_{44}^2 \quad (3)$$

According to Eq. (3), the pH (X_4) had the greatest effect on the

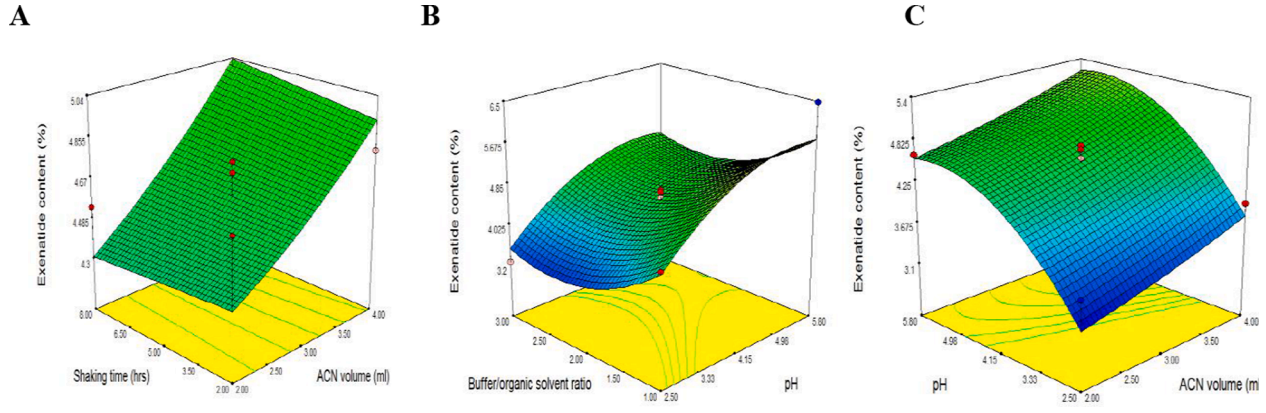

Fig. 1. Response surface plots indicating the effects of interaction between (**A**); Shaking time and ACN volume (**B**); buffer/organic solvent ratio and pH, and (**C**); pH and ACN volume.

response Y (exenatide content). The quadratic conditions of X_{22} and X_{44} had a significant effect on the response, as shown by their coefficients.

Counterplot and response surface plots are powerful tools that graphically show the relationship between the independent variables and the response (the exenatide content of the PLGA microspheres). Each plot represents the interaction of two variables, while the third variable is kept constant at its middle-level value.

The response surface plots (Fig. 1(A)) show that the shaking time had no significant effect on the measured exenatide content, whereas the buffer/organic solvent ratio, pH, and ACN volume did have a significant effect on it.

In Fig. 1(B), the response surface plot shows a curvature manner for the buffer/organic solvent ratio and pH of buffer axes which can be due to the statistical significance (p-value <0.05) of the quadratic coefficient of them in the model. As observed, increasing the buffer/organic solvent ratio from 1 to 2 resulted in a reduced response to a minimal level, followed by a slight increase for the buffer/organic solvent ratio >2. Likely, the solubility of the polymer in the mixture decreases with an increasing buffer volume fraction and likely resulted in co-precipitation of the peptide and polymer.

We found that with increasing ACN volume, the amount of extracted exenatide increased (Fig. 1(C)). The solubility of PLGA in a solvent depends on various factors such as the molecular weight and lactide to glycolide ratio of the polymer and temperature [41,42]. It is important to note that the optimal amount of ACN may depend on the specific formulation and properties of the microspheres, so it is important to optimize the extraction conditions for each individual case.

Fig. 1(C) clearly shows that with increasing the pH of the buffer from 2.5 to 5.8, the response increased to reach a plateau at pH 4.5. Exenatide has a pI value of 4.86 [24] and therefore, at pH 2.5 the peptide has the highest aqueous solubility, and thus the highest response would be expected. However, this was not observed. At pH lower than the pI, the peptide possesses a positive charge, and the interaction between the protonated amine and polarized carbonyl ester groups in PLGA may cause co-precipitation of peptide with the polymer. This finding is consistent with other studies that have reported the highest interaction between a peptide and PLGA at low pH, leading to the sorption of the peptide to the polymer [43,44]. Moreover, it has been shown that the stability of exenatide is highest at a pH of 4.5–5.5 [45].

3.3. Validation of the model

In order to maximize the response, by solving Eq. (3) and analyzing the response surface plots, the optimum level of ACN concentration, pH, buffer: organic solvent, and shaking time were determined to be 4 ml, 5.8, 1, and 5.6 hrs, respectively. At these conditions, the predicted exenatide content assay was calculated to be 6.1%.

Three experiments were performed using the identified statistically optimum levels. The maximum determined exenatide content was $6.4 \pm 0.0\%$, indicating a very good agreement with the predicted value (6.1%) which confirms the validity of the model. This means that encapsulation efficiency was 70.3 %.

Finally, in order to further validate the optimized model, the exenatide content in the marketed microspheres (Bydureon®) was determined using the optimized levels of the different variables. The determined Exenatide content of Bydureon® was $4.9 \pm 0.1\%$, which in excellent agreement with the claim on the label of Bydureon® (5.0%) [30].

4. Conclusion

The present study was focused on the development of an assay to determine the actual exenatide content in PLGA microspheres. After initial screening of six factors via FFD, a BBD approach was applied for optimization of the extraction method of exenatide from the PLGA microspheres. The excellent agreement between the actual and the predicted exenatide content shows the validity of the model.

The optimized method described in this paper may be useful for development of generic exenatide loaded-microsphere formulations. The developed method can also be used to determine the loading of other peptides, low molecular weight drugs as well as proteins and nucleic acid-based drugs in PLGA microspheres. Due to the reproducibility and robustness of the method, it is suitable for laboratory and industrial uses for exenatide microspheres as well as other PLGA-based long-acting release formulations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2023.09.016.

References

[1] N. Gulati, H. Gupta, Parenteral drug delivery: a review, Recent Pat. Drug Deliv. Formul. 5 (2) (2011) 133–145.
[2] J. Shen, D.J. Burgess, Accelerated in-vitro release testing methods for extended-release parenteral dosage forms, J. Pharm. Pharmacol. 64 (7) (2012) 986–996.
[3] C. Wischke, S.P. Schwendeman, Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles, Int. J. Pharm. 364 (2) (2008) 298–327.
[4] F. Ramazani, W. Chen, C.F. van Nostrum, G. Storm, F. Kiessling, T. Lammers, et al., Strategies for encapsulation of small hydrophilic and amphiphilic drugs in PLGA microspheres: state-of-the-art and challenges, Int. J. Pharm. 499 (1–2) (2016) 358–367, https://doi.org/10.1016/j.ijpharm.2016.01.020.
[5] J.M. Anderson, M.S. Shive, Biodegradation and biocompatibility of PLA and PLGA microspheres, Adv. Drug Deliv. Rev. 28 (1) (1997) 5–24.
[6] H. Keles, A. Naylor, F. Clegg, C. Sammon, Investigation of factors influencing the hydrolytic degradation of single PLGA microparticles, Polym. Degrad. Stab. 119 (2015) 228–241.
[7] A. Butreddy, R.P. Gaddam, N. Kommineni, N. Dudhipala, C. Voshavar, PLGA/PLA-based long-acting injectable depot microspheres in clinical use: production and characterization overview for protein/peptide delivery, Int. J. Mol. Sci. 22 (16) (2021) 8884.
[8] D.S. Pisal, M.P. Kosloski, S.V. Balu-Iyer, Delivery of therapeutic proteins, J. Pharm. Sci. 99 (6) (2010) 2557–2575.
[9] N. Teekamp, L.F. Duque, H.W. Frijlink, W.L. Hinrichs, P. Olinga, Production methods and stabilization strategies for polymer-based nanoparticles and microparticles for parenteral delivery of peptides and proteins, Expert Opin. Drug Deliv. 12 (8) (2015) 1311–1331.
[10] N.U. Khaliq, D. Chobisa, C.A. Richard, M.R. Swinney, Y. Yeo, Engineering microenvironment of biodegradable polyester systems for drug stability and release control, Ther. Deliv. 12 (1) (2021) 37–54.
[11] M. Alagusundaram, M.S. Chetty, K. Umashankari, A.V. Badarinath, C. Lavanya, S. Ramkanth, Microspheres as a novel drug delivery system: a review, Int J Chem Tech Res. 1 (3) (2009) 526–534.
[12] V.K. Nikam, V. Gudsoorkar, S. Hiremath, R. Dolas, V. Kashid, Microspheres-a novel drug delivery system: an overview, Int. J. Pharm. Chem. Sci. 1 (1) (2012).
[13] A. Namdev, R. Issarani, M. Khinchi, Microsphere as a novel drug delivery system: a review, Asian J. Pharm. Res. Dev. (2015) 15–24.
[14] S. Mao, C. Guo, Y. Shi, L.C. Li, Recent advances in polymeric microspheres for parenteral drug delivery–part 1, Expert Opin. Drug Deliv. 9 (9) (2012) 1161–1176.
[15] A. Rawat, U. Bhardwaj, D.J. Burgess, Comparison of in vitro–in vivo release of Risperdal® Consta® microspheres, Int. J. Pharm. 434 (1–2) (2012) 115–121.
[16] J.V. Andhariya, D.J. Burgess, Recent advances in testing of microsphere drug delivery systems, Expert Opin. Drug Deliv. 13 (4) (2016) 593–608, https://doi.org/10.1517/17425247.2016.1134484.
[17] M. Bragagni, M.E. Gil-Alegre, P. Mura, M. Cirri, C. Ghelardini, L.D.C. Mannelli, Improving the therapeutic efficacy of prilocaine by PLGA microparticles:

preparation, characterization and in vivo evaluation, Int. J. Pharm. 547 (1–2) (2018) 24–30.
[18] T. Schönbrodt, S. Mohl, G. Winter, G. Reich, NIR spectroscopy—a non-destructive analytical tool for protein quantification within lipid implants, J. Control. Release 114 (2) (2006) 261–267.
[19] M. Allahyari, E. Mohit, Peptide/protein vaccine delivery system based on PLGA particles, Hum. Vaccin. Immunother. 12 (3) (2016) 806–828.
[20] C. Zhu, T. Peng, D. Huang, D. Feng, X. Wang, X. Pan, et al., Formation mechanism, in vitro and in vivo evaluation of dimpled exenatide loaded PLGA microparticles prepared by ultra-fine particle processing system, AAPS PharmSciTech 20 (2) (2019) 1–10.
[21] L. Meinel, O.E. Illi, J. Zapf, M. Malfanti, H.P. Merkle, B. Gander, Stabilizing insulin-like growth factor-I in poly (D, L-lactide-co-glycolide) microspheres, J. Control. Release 70 (1–2) (2001) 193–202.
[22] H. Sah, A new strategy to determine the actual protein content of poly (lactide-co-glycolide) microspheres, J. Pharm. Sci. 86 (11) (1997) 1315–1318.
[23] J. Wang, B.M. Wang, S.P. Schwendeman, Characterization of the initial burst release of a model peptide from poly (D, L-lactide-co-glycolide) microspheres, J. Control. Release 82 (2–3) (2002) 289–307.
[24] M.B. DeYoung, L. MacConell, V. Sarin, M. Trautmann, P. Herbert, Encapsulation of exenatide in poly-(D, L-lactide-co-glycolide) microspheres produced an investigational long-acting once-weekly formulation for type 2 diabetes, Diabetes Technol. Ther. 13 (11) (2011) 1145–1154.
[25] M. Yu, M.M. Benjamin, S. Srinivasan, E.E. Morin, E.I. Shishatskaya, S. P. Schwendeman, et al., Battle of GLP-1 delivery technologies, Adv. Drug Deliv. Rev. 130 (2018) 113–130.
[26] M. Fineman, S. Flanagan, K. Taylor, M. Aisporna, L.Z. Shen, K.F. Mace, et al., Pharmacokinetics and pharmacodynamics of exenatide extended-release after single and multiple dosing, Clin. Pharmacokinet. 50 (1) (2011) 65–74.
[27] R.R. Holman, M.A. Bethel, R.J. Mentz, V.P. Thompson, Y. Lokhnygina, J.B. Buse, et al., Effects of once-weekly exenatide on cardiovascular outcomes in type 2 diabetes, N. Engl. J. Med. 377 (13) (2017) 1228–1239.
[28] Y. Wang, T. Sun, Y. Zhang, B. Chaurasiya, L. Huang, X. Liu, et al., Exenatide loaded PLGA microspheres for long-acting antidiabetic therapy: preparation, characterization, pharmacokinetics and pharmacodynamics, RSC Adv. 6 (44) (2016) 37452–37462.
[29] F. Qi, J. Wu, D. Hao, T. Yang, Y. Ren, G. Ma, et al., Comparative studies on the influences of primary emulsion preparation on properties of uniform-sized exenatide-loaded PLGA microspheres, Pharm. Res. 31 (6) (2014) 1566–1574.
[30] T. Li, A. Chandrashekar, A. Beig, J. Walker, J.K. Hong, A. Benet, et al., Characterization of attributes and in vitro performance of exenatide-loaded PLGA long-acting release microspheres, Eur. J. Pharm. Biopharm. 158 (2021) 401–409.
[31] Z. Rahman, A.S. Zidan, M.A. Khan, Non-destructive methods of characterization of risperidone solid lipid nanoparticles, Eur. J. Pharm. Biopharm. 76 (1) (2010) 127–137.
[32] I.M. Fukuda, C.F.F. Pinto, C.S. Moreira, A.M. Saviano, F.R. Lourenço, Design of experiments (DoE) applied to pharmaceutical and analytical quality by design (QbD), Brazilian J. Pharm. Sci. 54 (2018).
[33] S. Beg, S. Swain, M. Rahman, M.S. Hasnain, S.S. Imam, Application of design of experiments (DoE) in pharmaceutical product and process optimization, Pharmaceutical quality by design. Elsevier (2019) 43–64.
[34] S. Honary, P. Ebrahimi, R. Hadianamrei, Optimization of particle size and encapsulation efficiency of vancomycin nanoparticles by response surface methodology, Pharm. Dev. Technol. 19 (8) (2014) 987–998.
[35] F. Khoshayand, S. Goodarzi, A.R. Shahverdi, M.R. Khoshayand, Optimization of culture conditions for fermentation of soymilk using Lactobacillus casei by response surface methodology, Probiotics Antimicrob. Proteins 3 (3) (2011) 159–167.
[36] R.H. Myers, D.C. Montgomery, C.M. Anderson-Cook, Response surface methodology: process and product optimization using designed experiments, John Wiley & Sons, 2016.
[37] M. Shirangi, W.E. Hennink, G.W. Somsen, C.F. Van Nostrum, Identification and assessment of octreotide acylation in polyester microspheres by LC–MS/MS, Pharm. Res. 32 (9) (2015) 3044–3054.
[38] R. Liang, R. Zhang, X. Li, A. Wang, D. Chen, K. Sun, et al., Stability of exenatide in poly (D, L-lactide-co-glycolide) solutions: a simplified investigation on the peptide degradation by the polymer, Eur. J. Pharm. Sci. 50 (3–4) (2013) 502–510.
[39] Y. Zhang, A.M. Sophocleous, S.P. Schwendeman, Inhibition of peptide acylation in PLGA microspheres with water-soluble divalent cationic salts, Pharm. Res. 26 (8) (2009) 1986–1994.
[40] A. Rai, B. Mohanty, R. Bhargava, Supercritical extraction of sunflower oil: a central composite design for extraction variables, Food Chem. 192 (2016) 647–659.
[41] K. Park, S. Skidmore, J. Hadar, J. Garner, H. Park, A. Otte, et al., Injectable, long-acting PLGA formulations: analyzing PLGA and understanding microparticle formation, J. Control. Release 304 (2019) 125–134, https://doi.org/10.1016/j.jconrel.2019.05.003.
[42] H.K. Makadia, S.J. Siegel, Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier, Polymers 3 (3) (2011) 1377–1397.
[43] D.H. Na, DeLuca pp., PEGylation of octreotide: I. Separation of positional isomers and stability against acylation by poly (D, L-lactide-co-glycolide), Pharm. Res. 22 (2005) 736–742.
[44] A.M. Sophocleous, Y. Zhang, S.P. Schwendeman, A new class of inhibitors of peptide sorption and acylation in PLGA, J. Control. Release 137 (3) (2009) 179–184, https://doi.org/10.1016/j.jconrel.2009.03.006.
[45] A. Benet, T. Halseth, J. Kang, A. Kim, R. Ackermann, S. Srinivasan, et al., The effects of pH and excipients on exenatide stability in solution, Pharmaceutics 13 (8) (2021) 1263.