



Development of Level A *in vitro-in vivo* correlations for peptide loaded PLGA microspheres

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ARTICLE INFO

Keywords:

Leuprolide acetate
PLGA
Peptide microspheres
In vitro-in vivo correlation (IVIVC)
Compositionally equivalent
Release testing

ABSTRACT

Peptide loaded PLGA microsphere products are more complex in terms of manufacturing, drug release characteristics as well as release mechanism compared to small molecule loaded PLGA microsphere products. This is due to the complex structure of peptides, their hydrophilicity, charged state, large size and potential for instability. Moreover, therapeutic peptides are highly potent and therefore, any unintended change in the microsphere release profile may lead to undesirable side effects and toxicity. Accordingly, the objectives of the present work were: 1) to evaluate the impact of minor manufacturing changes on the quality and performance of peptide microspheres; and 2) to investigate the feasibility of developing Level A *in vitro-in vivo* correlations (IVIVCs) for peptide microspheres. Compositionally equivalent leuprolide acetate (LA) microspheres prepared with minor manufacturing changes (solvent system/homogenization speed) showed significant differences in their physicochemical properties (such as pore size, total porosity, particle size and surface distribution of peptide on the prepared microspheres). This, in turn, resulted in significant alteration in the release characteristics. Peptide-polymer interaction, *in vitro* degradation and microsphere morphology studies were conducted to facilitate understanding of the differences in the drug release characteristics. A rabbit model was used to determine the pharmacokinetic profiles of all the prepared formulations. The obtained *in vivo* release profiles showed the same rank order as the *in vitro* release profiles but with low burst release and overall faster *in vivo* release rates. The low *in vivo* burst release is considered to be due to the masking effect of the absorption phase from the intramuscular site, and this complicated the development of an IVIVC. Despite these challenges, an affirmative Level A IVIVC over the entire release profile was successfully developed in a rabbit model for peptide microspheres for the first time. The developed IVIVC was also predictive of the RLD product, Lupron Depot®. This work highlights the feasibility of developing IVIVCs for complex parenteral drug products such as peptide microspheres. In conclusion, these results indicate the sensitivity of peptide release, and hence, the safety and efficacy of highly potent peptide microspheres, to minor manufacturing changes. Accordingly, development of IVIVCs for such complex drug products is highly desirable.

1. Introduction

As per the U.S. FDA guidance [1], an *in vitro-in vivo* correlation (IVIVC) is a predictive mathematical model describing the relationship between an *in vitro* property (e.g. rate or extent of drug release) of a dosage form and a relevant *in vivo* response (e.g. plasma drug concentration or amount of drug absorbed vs time). Despite the successful commercialization of various types of extended release microsphere drug products and their increasing popularity in the past few decades [2], there have been few reports on the development of IVIVCs. Most of

the reported literature is “proof-of-concept” research demonstrating the possibility of developing IVIVCs for microspheres [3–9]. This is because the development of IVIVCs for long-acting polymeric microspheres is very challenging principally due to their complex drug release characteristics and the lack of standard bio-predictive *in vitro* release testing methods [10–14] as well as the lack of regulatory guidance. Despite these challenges, our group has recently developed a 1:1 linear correlation, Level A [1] IVIVC (the most affirmative type of IVIVC with the potential to be used to obtain biowaivers) for microsphere drug products containing a variety of small molecules [15,16]. These

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<https://doi.org/10.1016/j.jconrel.2019.07.013>

Received 20 March 2019; Received in revised form 29 June 2019; Accepted 9 July 2019

Available online 10 July 2019

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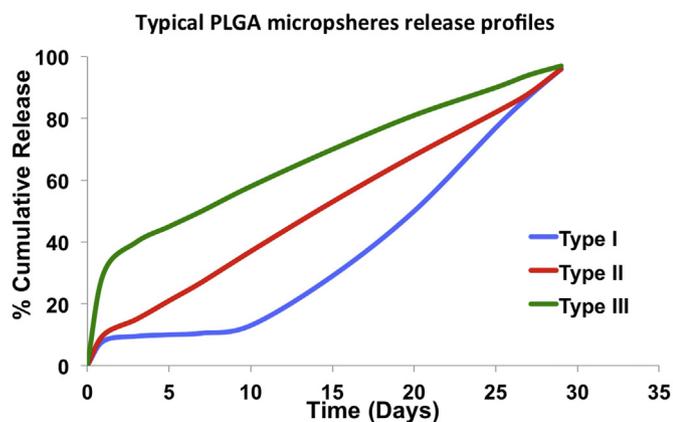


Fig. 1. Schematic representation of typically observed drug release profiles of PLGA microspheres.

Type I – Low burst release phase followed by lag phase and secondary release phase.

Type II – Low burst release phase followed by secondary release phase.

Type III – High burst release phase followed by secondary release phase.

microsphere products had tri-phasic and bi-phasic *in vitro* release profiles with and without a lag phase, respectively but with low burst release (*i.e.* Type-I and Type-II release profiles, Fig. 1). Fig. 1 is representative of the different types of release profiles typically observed with PLGA microsphere products. Compared to the release Types I and II, the release Type III PLGA microspheres have a high burst release followed by slow and continuous release. To further explore the concept and achieve a comprehensive understanding of the development of IVIVCs for PLGA microspheres, it was considered essential to investigate the feasibility of developing IVIVCs for the release Type III microsphere drug products. Typically, microspheres containing hydrophilic drug molecules (peptides and small molecules) tend to have higher burst release (Type III). Moreover, peptides have complex structure, charged state and larger size compared to small molecules and therefore, peptide microspheres have more complex release mechanisms compared to small molecule microspheres. This may result in variations in drug release under *in vivo* conditions compared to *in vitro* conditions, complicating the development of IVIVCs for peptide microspheres. There have been no reports to date on the development of Level A IVIVCs for compositionally equivalent peptide microspheres.

Peptide microsphere products are also more complex in terms of manufacturing as a result of the hydrophilic nature, large size and relative instability of peptides. Moreover, due to differences in the physicochemical properties of peptides and small molecules, the impact of minor manufacturing changes on the quality and performance of peptide microspheres may be more or less significant compared to small molecule microspheres. In addition, therapeutic peptides are highly potent and hence, any unintended minor changes in the release profiles may lead to undesirable side effects and toxicity. Accordingly, it is crucial to understand the impact of minor manufacturing changes on the physicochemical properties (quality) and release characteristics (performance) of compositionally equivalent peptide microsphere drug products.

The therapeutic peptide, leuprolide acetate (LA) was chosen as the model peptide since it is commercially available in the microsphere drug product, Lupron Depot® (RLD). This product is off patent, and accordingly generic companies may be actively seeking to develop generic versions. Therefore, it is critical for the industry as well as the regulatory authorities to understand the impact of manufacturing differences on the quality and performance of LA microsphere drug products. An additional aspect is that this product is prepared using a low molecular weight (M.wt.) PLGA [17–19], and there have been no studies investigating the impact of minor manufacturing changes on the

performance of such microspheres. Since this product contains positively charged peptide and the polymer is negatively charged at pH 7.4, there is potential for charge–charge interaction to affect drug release [18]. Hence, this study will provide important insights into the impact of manufacturing differences on the release mechanism of such complex microsphere products.

The objectives of the present work were to evaluate the impact of minor manufacturing changes on the physicochemical properties and drug release characteristics as well as to investigate the feasibility of developing a Level A IVIVC for compositionally equivalent parenteral microsphere products containing a peptide drug. Leuprolide acetate (LA) was chosen as the model peptide. Four compositionally equivalent LA microspheres were prepared with manufacturing differences and characterized for various physicochemical properties (such as drug loading, particle size, porosity, morphology and M.wt.). The *in vitro* release characteristics of the prepared LA microsphere formulations and the RLD product were determined using the developed *in vitro* release testing method. The pharmacokinetic profiles of the LA microspheres were investigated using a rabbit model. The *in vivo* profiles were deconvoluted using the Loo-Riegelman method and compared with the respective *in vitro* release profiles to investigate the possibility of establishing an IVIVC.

2. Material and methods

2.1. Materials

PLGA (Resomer® Select 7525 DLG 2CA) was purchased from Evonik (Birmingham, AL). Leuprolide Acetate (LA) and reference standard (*i.e.* LHRH Acetate salt) were purchased from Bachem Americas, Inc. (Torrance, CA). Poly (vinyl alcohol) (PVA, M.wt. 30–70 kDa) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade Methylene chloride (DCM), methanol (MeOH), acetonitrile (ACN) and ACS-grade dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). LC-MS grade 0.1% v/v formic acid in water, 0.1% v/v ammonium acetate in water and acetonitrile were purchased from VWR (Radnor, PA). Milli-Q® water was used for all studies. All other chemicals were obtained commercially as analytical-grade reagents.

2.2. Preparation of leuprolide acetate microspheres

PLGA (M.wt. ~15KDa) with a similar molecular weight as that used in the commercial product, Lupron Depot®, was used to formulate compositionally equivalent LA microsphere formulations using different manufacturing processes. Briefly, 200 mg of PLGA was dissolved in methylene chloride (45%, w/v). LA is poorly soluble in methylene chloride and accordingly, a co-solvent (such as methanol and DMSO) was used to facilitate the dissolution of LA (~30 mg). The LA solution was then added to the polymer solution to form the organic phase and mixed *via* vortexing. The organic phase containing both the polymer and drug was then added at once into a 100 mL of 0.35% (w/v) PVA solution (0.22 μm membrane filtered) to form an oil-in-water (o/w) emulsion using a homogenizer (IKA® Works, Inc.) set at 14,000 rpm for 1 min. Leuprolide acetate microspheres with large particle size were prepared using a lower emulsification/size reduction force (homogenizer set at 9000 rpm for 1 min). The resultant o/w emulsion was stirred at 400 rpm for 3 h under vacuum at room temperature to allow microsphere solidification and solvent evaporation. The resultant microspheres were collected by centrifugation; washed using distilled water and freeze dried using a vacuum manifold.

2.3. Characterization of LA microspheres

2.3.1. High performance liquid chromatography (HPLC) analysis

The quantification of LA was conducted using an Agilent HPLC

Table 1
HPLC gradient elution method for leuprolide acetate analysis.

Time (min)	Flow rate (mL/min)	%A	%B
0	1.0	90	10
10	1.0	80	20
12	1.0	90	10
15	1.0	90	10

system (series 1200) with a UV absorbance detector set at 220 nm (release test samples)/280 nm (drug loading test samples). The mobile phase consisting of 0.1% (v/v) tri-fluoroacetic acid in water (A) and acetonitrile (B) was used for separation of LA through gradient elution (Table 1) at 30 °C. A Kinetex® EVO C18 column (250 × 4.6 mm, 5 μm, 100 Å, Phenomenex) was used as the stationary phase. The sample injection volume was 20 μL for drug loading and 50 μL for *in vitro* release testing sample analysis. The chromatographs were analyzed using an Agilent OpenLAB chromatography data system.

2.3.2. Drug loading

The LA microspheres (~5 mg) were weighed and transferred into 10 mL volumetric flasks. DMSO (2 mL) was added into the volumetric flasks and the samples were sonicated until all particles were dissolved. Methanol was used to dilute the samples. The solutions were filtered (Millex® HV, 0.22 μm PVDF syringe filter) and the LA concentrations were determined via the validated HPLC assay as described above. All the measurements were conducted in triplicate and the results are reported as the mean ± SD. Drug loading was calculated as:

$$\text{Drug Loading (\%)} = \frac{\text{weight of drug entrapped}}{\text{weight of microspheres analyzed}} \times 100$$

2.3.3. Particle size and size distribution

Particle size and particle size distribution of the LA microspheres were measured using an AccuSizer autodiluter particle sizing system (Nicom, Santa Barbara, CA). Briefly, the microspheres were dispersed in a filtered 0.1% (w/v) PVA solution in water by vortex mixing to ensure good dispersion, sonicated and then particle size analysis was conducted. All the measurements were conducted in triplicate and the results are reported as the mean size ± SD.

2.3.4. Morphology

The morphology of the LA microspheres was analyzed using scanning electron microscopy (SEM). Briefly, dry microspheres were mounted on carbon taped aluminum stubs and sputter coated with gold under an argon evaporator at high vacuum. The samples were then observed using SEM (NanoSEM 450, Nova).

2.3.5. Porosity

The porosity of the LA microspheres was determined using a Mercury Porosimeter (AutoPore IV 9500, Micromeritics). Briefly, approximately 200 mg of LA microspheres were introduced into the porosimeter and tested at a mercury filling pressure starting at 0.53 psi. Total % porosity and average pore diameter were recorded.

$$\text{Porosity (\%)} = \left(1 - \frac{\text{Bulk density}}{\text{Apparent (skeletal) density}} \right) \times 100$$

2.3.6. Differential scanning calorimeter (DSC) analysis

The glass transition temperatures (T_g) of the LA microspheres were analyzed using a modulated temperature differential scanning calorimeter (MTDSC) (TA Instruments Q2000). Briefly, weighed quantity (~5–6 mg) of each sample was transferred to standard aluminum pans and sealed with aluminum lids. The DSC experiment was performed using a 2 °C/min heating rate and a modulation amplitude of ± 0.82 °C with an

80 s modulation period. All samples were subjected to a heat/cool/heat cycle. The results were analyzed by using TA analysis software, and the T_g was determined as the glass transition midpoint in the reversing signal.

2.3.7. Molecular weight

The molecular weight of the microspheres was determined by gel permeation chromatography (GPC; Waters) with an evaporative light scattering detector (ELSD). The mobile phase was tetrahydrofuran (THF) with a flow rate of 2 mL/min at 40 °C. Samples were dissolved in THF at a concentration of 1 mg/mL and filtered (0.45 μm filter, Millipore, USA) before injection into the GPC system. The data collection and analysis were performed using Waters Millennium software. Polystyrene standards (2000, 900, 824, 400, 200, 110, 43, 18.80, 17.60, 6.93, 2.61, 0.98 kDa) were used for calibration and weight average molecular weights (M_w) were calculated. All measurements were conducted in triplicate and the results are reported as the mean ± SD.

2.4. Peptide-polymer interaction study

FT-IR spectroscopy was performed using a Nicolet FTIR (iS5 FTIR, Thermo Scientific) spectrometer attached with an attenuated total reflectance (ATR) accessory. Briefly, the samples were placed on the crystal window (Germanium) and compressed lightly using a pressure clamp. Spectra were recorded over a range of 400–4000 cm⁻¹ with a resolution of 4 cm⁻¹, for 128 parallel scans and evaluated for any shifts in the functional peaks. Data analysis was performed using Omnic software (Thermo Nicolet Corporation). The physical mixture was prepared using an accurately weighted quantity of drug and polymer in a ratio same as that of the drug loading of prepared LA microspheres (Polymer: drug, 92:8% w/w).

2.5. In vitro release studies

In vitro release testing of the LA microspheres was conducted using a developed sample-and-separate method at 37 °C. Briefly, 6 mg of microspheres were dispersed in 1 mL of phosphate buffered saline (PBS containing 0.02% (v/v) Tween 20 and 0.02% (w/v) sodium azide, 33 mM, pH 7.4) in low protein binding eppendorf tubes mounted on a rotator set at 100 rpm and incubated at 37 °C. At pre-determined time intervals, release media was collected and centrifuged at 3000 rpm for 3 min. Supernatants (0.3 mL) were filtered through 0.22 μm filters and analyzed via HPLC. The release media was replaced with fresh media at each sampling time point. Media replacement during release testing was taken into account in the calculation of the % cumulative release. All drug release tests were conducted in triplicate and the results are reported as the mean % cumulative release ± SD.

2.6. In vitro degradation studies

An exhaustive *in vitro* degradation study of the LA microspheres was conducted similar to the *in vitro* release testing method. At pre-determined time points, microsphere samples incubated under release testing conditions were collected, washed, lyophilized and evaluated for polymer molecular weight using gel permeation chromatography (GPC) and morphology using scanning electron microscopy (SEM) as mentioned above.

2.7. In vivo release studies

The *in vivo* release characteristics of the LA microspheres were investigated using a rabbit model. Briefly, male rabbits (New Zealand White, n = 6) weighing approximately 3 to 4 kg were randomly assigned to each treatment group *i.e.* formulation. The LA microspheres were suspended in the diluent used for dispersion of the commercial

product Lupron Depot® and injected into the rabbit hind leg thigh muscles at a dose of 2.14 mg/kg. In addition, a pharmacokinetic study of the LA solution in saline (dose: 0.025 mg/kg, *i.v.*) injected via the intravenous route was conducted ($n = 6$) to determine the PK parameters of LA in rabbit. Blood samples were collected from the marginal ear veins at predefined time intervals. The collected blood samples were centrifuged at 14,500 rpm for 5 min to separate out the plasma. The plasma was collected and stored at -80°C until analysis. The animal study protocol was reviewed and approved by the University of Connecticut's Institutional Animal Care and Use Committee (IACUC) prior to the beginning of the experiments.

2.8. Plasma sample analysis

LA ((Des-Gly₁₀, D-Leu₆, Pro-NHEt₉)-LHRH, M.wt. 1209.42 Da) was extracted from plasma samples using a protein precipitation extraction method. LHRH ((Des-Gly₁₀, D-Ala₆, Pro-NHEt₉)-LHRH, M.wt. 1167.34 Da) acetate salt was used as the internal standard (IS). Briefly, 100 μL of plasma samples were mixed with either 100 μL of LA solution in diluent (0.1% formic acid in water: acetonitrile (90:10, %v/v)) for the preparation of calibration standards (1, 2.5, 5, 10, 15, 20 and 30 ng/mL) or 100 μL of diluent for study samples. All the samples were then spiked with 20 μL of the IS solution (200 ng/mL in methanol). Ice-cold acetonitrile was then added in a stepwise fashion (two-steps, each of 500 μL) to the pre-treated plasma samples and vortex-mixed for 10 min at 2500 rpm followed by centrifugation at 14,500 rpm (4°C) for 8 min. The supernatants were transferred to the low-protein binding polypropylene (PP) centrifuge tubes and dried under nitrogen at 40°C . The dry residues were then reconstituted in 100 μL of diluent (0.1% formic acid in water/acetonitrile, 90:10%v/v). 25 μL of the reconstituted solution was used for LC-MS/MS analysis. Please note that during the entire preparation process, plasma samples were processed in the ice bath before the collection of supernatant samples for drying.

The LC-MS/MS system consisted of an Agilent HP-1100 LC system and a TSQ Quantum Ultra Mass Spectrometer (Waters) with an electrospray ionization (ESI) ion source. Chromatographic separations were carried out on a Kinetex EVO C18 column ($50 \times 2.1 \text{ mm}$, $2.6 \mu\text{m}$, 100 \AA) through gradient elution (Table 2) at 35°C . The following MS detection parameters were used: 4400 V electrospray voltage, 320°C capillary temperature, and 35 V collision energy. Detection of ions was conducted in the positive-ion selected reaction monitoring mode with the following transitions in a single reaction monitoring (SRM) mode: m/z 605.5 to 249.1 for leuprolide acetate, and 584.67 to 249.1 for the IS. The injection volume was 25 μL . The data acquisition was ascertained by Xcalibur software. Calibration curves were established on each day when analysis was conducted and showed good linearity with correlation coefficients > 0.99 . The lowest limit of quantification (LLOQ) for LA was 1 ng/mL and the mean recovery of plasma samples from low to high concentrations of LA was $> 90\%$. The inter- and intraday variations of the three different concentrations of LA (1, 10, and 30 ng/mL) were $< 15\%$.

Table 2

HPLC gradient method for the determination of leuprolide acetate content in rabbit plasma.

Time (min)	Flow rate (mL/min)	0.1% formic Acid/0.1% ammonium acetate (21/, v/v), %	ACN, %
0	0.3	95	5
5	0.3	40	60
8	0.3	40	60
9	0.3	95	5
12	0.3	95	5

2.9. Pharmacokinetic (PK) analysis and the development of an IVIVC

The development of IVIVC for the prepared LA microspheres was performed using a two stage deconvolution approach following the same principles as detailed in the U.S. FDA guidance on the development of IVIVC for extended release oral dosage forms [1]. Briefly, the *in vivo* plasma profiles of LA microspheres were deconvoluted using the Loo-Riegelman method [12,20]. Standard errors are not shown in the deconvoluted *in vivo* absorption profiles because the average plasma concentration values were used. The fraction absorbed *in vivo* was calculated as below:

$$\frac{Ab}{Ab_{\infty}} = \frac{C_p + C_t + K_{10}(AUC)_0^t}{K_{10}(AUC)_0^{\infty}}$$

C_p , C_t , K_{10} , and AUC are the drug concentration in the central compartment (plasma), apparent tissue compartment concentration, elimination rate constant and area under the plasma vs time curve, respectively. The distributive and elimination micro rate constants (k_{12} , k_{21} and k_{10}) that are necessary for calculating C_t , and the total fraction absorbed at time t , were calculated using a two compartment model, PK analysis tool, WinNonlin® 6.4 (Pharsight, Certara Corporation, St. Louis, USA) from the plasma concentrations vs time profiles of LA solution after single intravenous administration in rabbits. Similarly, PK parameters of all the LA microsphere formulations were determined using non-compartmental analysis. The development and validation of the IVIVC for the LA microspheres were performed using the IVIVC toolkit, WinNonlin® 6.4 (Pharsight, Certara Corporation St. Louis, USA).

2.10. Statistical data analysis

Statistical analysis was performed to evaluate significant differences between different microsphere formulations using a paired student t -test. The level of significance was accepted at $p < .05$.

3. Results and discussion

3.1. Physicochemical properties of the LA microspheres

Four LA microsphere formulations (*i.e.* F1, F2, F3 and F4) with Q1/Q2 equivalency were prepared with minor manufacturing changes (such as cosolvents and homogenization speed) and evaluated for differences in physicochemical properties, if any. The RLD product (Lupron Depot® (one month)) was used as a control. As shown in Table 3, the drug loading of the RLD product was around 7% (w/w) and all the prepared LA microsphere formulations had similar drug loading of around 8% (w/w). This shows that the prepared LA microsphere formulations were Q1/Q2 equivalent despite minor changes in the manufacturing process.

The mean particle size and size distribution of the prepared LA microspheres and the RLD product were evaluated. As shown in Fig. 2, all the prepared LA microsphere formulations had larger particle size in terms of volume distribution and smaller or equal particle size in terms of population distribution when compared to the RLD product. This, in turn, indicates a wide size distribution (Fig. 2B) in all the prepared microsphere formulations compared to the RLD product. Moreover, formulations F1 and F3, prepared using similar homogenization speeds and time, did not show significant differences ($p > .05$) in the particle size despite the use of different solvent systems. The population mean size of F3 was lower than that of F1, although the two formulations had similar volume mean size. Accordingly, F3 had a wider size distribution and hence a higher span value than F1 in terms of population distribution. However, the observed difference in the population mean size of the two formulations was not statistically significant ($p > .05$). This indicates that the impact of the solvent system on droplet size during

Table 3
Drug loading of the RLD and the LA microspheres prepared with minor manufacturing changes.

Sample	Preparation parameters	Drug loading (% w/w)
RLD	–	6.88 ± 0.77
F1	Homogenization (DCM/methanol: 2.6/1, w/w)	8.36 ± 0.30
F2	Homogenization (DCM/methanol: 2.6/1, w/w) (large particle size)	8.55 ± 0.08
F3	Homogenization (DCM/DMSO: 3.7/1, w/w)	8.47 ± 0.14
F4	Homogenization (DCM/DMSO: 3.7/1, w/w) (large particle size)	8.14 ± 0.01

emulsification and of solvent evaporation rate during microsphere solidification on the final particle size was not significant ($p > .05$). However, formulations F2 and F4 had a larger mean particle size in terms of volume distribution compared to formulations F1 and F3 prepared using the same solvent system (Fig. 2). This is due to the use of lower homogenization speed during the emulsification process [21,22].

As shown in Fig. 3A, some irregular shapes (indicated by the green arrow) and a highly porous polymer matrix (indicated by the red arrows) were observed for the RLD product. On the other hand, the prepared LA microspheres showed a spherical shape with a smooth surface (Fig. 3B to E). Compared to formulations F1 and F2, formulations F3 and F4 showed a more porous structure, which may be due to the different solvent systems used in processing. Different solvent systems also lead to changes in the polymer precipitation rate. For example, PLGA is more soluble in DCM/DMSO compared to DCM/methanol. Moreover, DMSO has a higher boiling point and hence, will be removed slowly compared to methanol. Taken together, these differences will lead to relatively slower solidification into microspheres for formulations F3 and F4 compared to formulations F1 and F2, allowing more dynamic solvent exchange and entrapment inside the microspheres. The removal of the entrapped solvents during drying will create pores and hence, formulations F3 and F4 have more porous structures compared to formulations F1 and F2.

The observed differences in the porous structure of the prepared microsphere formulations were further confirmed and quantified by evaluation of the % porosity of the LA microsphere formulations. As shown in Table 4, overall differences in the total % porosity of the formulations were observed with F3 being the most porous and F2 being the least porous/more dense formulation. In agreement with the morphological characteristics (Fig. 2), formulations F3 and F4 had relatively higher % porosity compared to formulations F1 and F2, respectively.

Since LA is a peptide of relatively higher molecular weight/larger size compared to small molecules, it is reported that the pore diameter can also be a critical parameter controlling diffusion out of microspheres into the release medium [23]. Accordingly, the average pore diameter of the prepared LA microsphere formulations was

investigated. As shown in Table 4, significant differences ($p < .05$) in the average pore diameter of all the prepared formulations were observed with F2 having the smallest pore diameter and F3 having the largest pore diameter. The pore diameters of formulations F1 and F2 were smaller than F3, which shows that the differences in the solvent systems leads to differences in the pore diameter. In addition, the pore diameters of formulations F2 and F4 (smaller particle size microspheres) were smaller than those of formulations F1 and F3 (larger particle size microspheres), respectively. Similarly, the % porosity of formulations F2 and F4 was smaller than that of formulations F1 and F3, respectively. These results indicate that particle size, in addition to the solvent system, has the potential to alter the % porosity and pore diameter. It has been reported that faster solvent removal and polymer precipitation leads to the formation of large pores [24]. Since diffusion distance will be relatively shorter in small particles compared to larger particles, solvent exchange and solvent removal will be faster for small particles compared to large particles.

Please note that the F1 and F4 had similar % porosity as well as pore diameter, which may be the result of the combined and opposite effects of differences in their solvent systems (hence % porosity) as well as particle size (hence, pore size), as explained above.

As shown in Table 4, no significant differences in the T_g and polymer molecular weight of the prepared LA microsphere formulations were observed for the minor manufacturing changes investigated. However, the physicochemical properties of LA microspheres (such as particle size, morphology, pore size, and % porosity) are sensitive to the minor manufacturing differences.

3.2. *In vitro* release characteristics of the LA microspheres

The *in vitro* release testing method was developed by screening different methods and conditions (such as membrane dialysis vs sample and separate methods, buffer strength, media replacement), with the aim to achieve 100% cumulative drug release as well as to maintain peptide stability throughout the release study (data not shown). As shown in Fig. 4A, 100% drug release was achieved using the sample-and-separate *in vitro* release testing method. In addition, the method

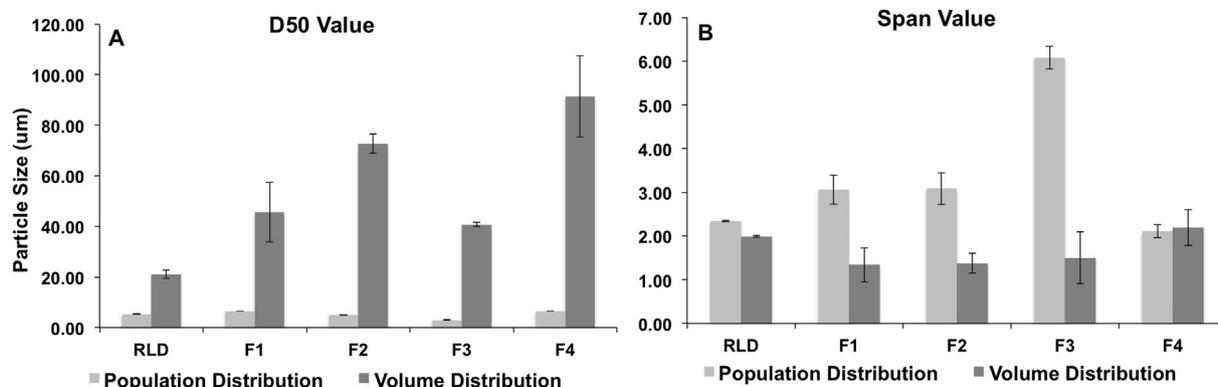


Fig. 2. Particle size and size distribution of the RLD product and LA microsphere formulations prepared using minor manufacturing differences. (A) D50 value; and (B) Span value. All values are expressed as mean ± SD ($n = 3$).

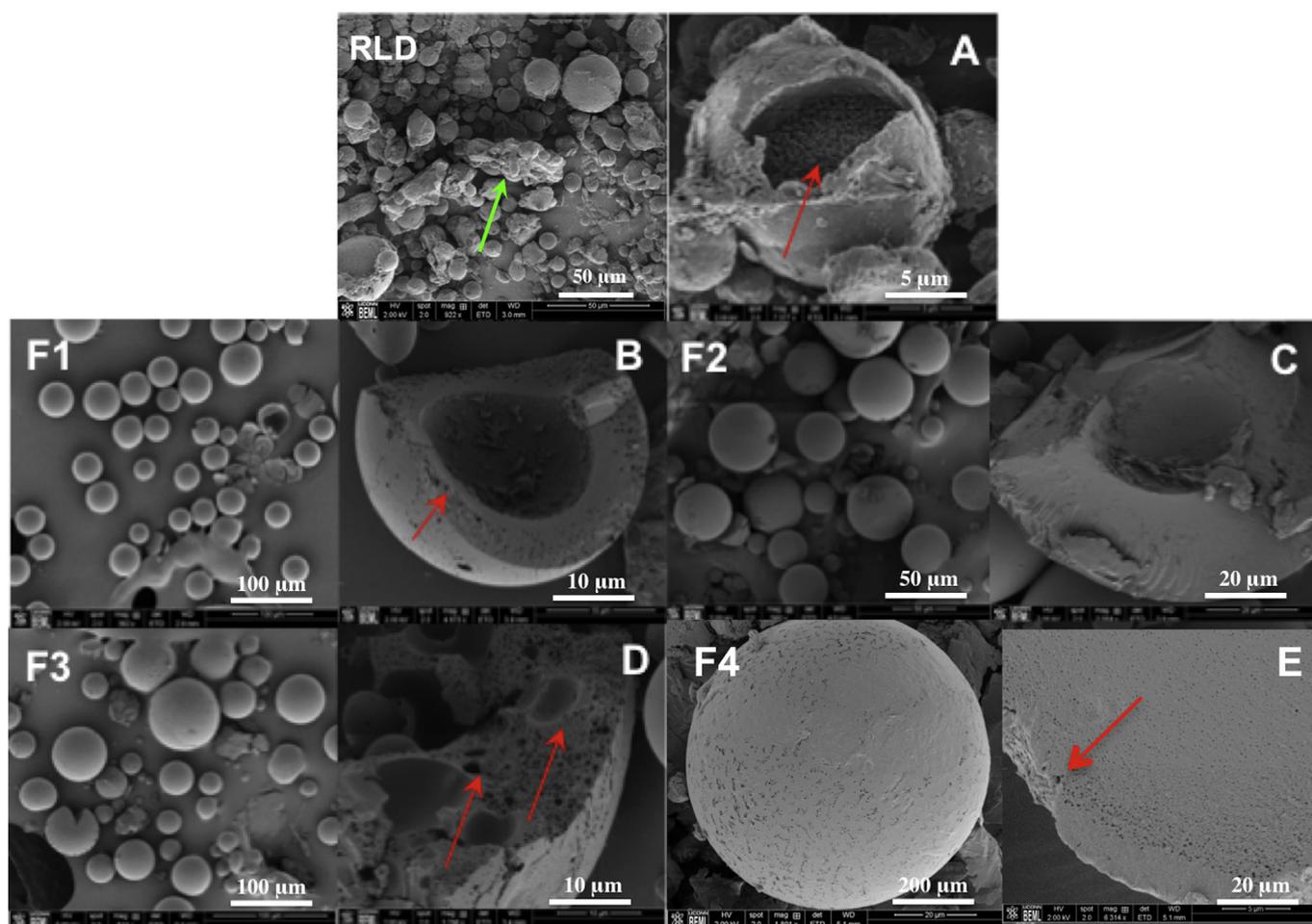


Fig. 3. SEM images of the RLD product and leuprolide acetate microspheres. Symbol: The green arrow points to the irregular shapes of microspheres and the red arrows point to pores in the microsphere matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Physicochemical properties of the prepared leuprolide acetate microsphere formulations.

Formulation	Porosity (%)	Avg. pore diameter (nm)	Tg (°C)	Molecular weight (D) (Mean ± SD)
F1	57.06	814.5	44.1	13,245 ± 173
F2	52.65	712.7	45.32	13,406 ± 41
F3	61.01	964	44.78	13,541 ± 22
F4	56.48	814.1	45.27	13,550 ± 60

had good reproducibility with < 5% RSD at all time points (Fig. 4A). As shown in Fig. 4B, the *in vitro* release method was also able to discriminate changes in the drug release characteristics of the LA microsphere formulations prepared using minor manufacturing changes as well as the RLD product. Overall, all formulations appeared to follow biphasic release characteristics with an initial burst release phase followed by an apparent zero-order slow release phase over a period of at least 40 days.

Differences in the drug release profiles, especially the burst release phase (from 10% to 39% in 24 h) were observed for the formulations. Variable burst release is a result of differences in the amount of surface associated drug, which may be due to one or the combination of the following factors: 1) higher solubility of PLGA in DCM/DMSO compared to DCM/MeOH; and 2) slower evaporation of DMSO compared to MeOH. These two factors result in slower microsphere solidification and hence, more drug diffusion onto the surface of formulations F3 and

F4 compared to F1 and F2. In addition, the higher solubility of LA in DMSO compared to MeOH leads to greater accumulation of drug onto the surface of formulations F3 and F4 during solvent exchange. Moreover, formulations F1 and F3 have small particle size compared to formulations F2 and F4, resulting in the higher surface area to volume ratio and hence, a greater amount of surface associated drug leading to higher burst release.

In addition to the burst release phase, differences in the release rate of all the LA microspheres were observed over the initial 15-day period (Fig. 4B). Formulation F3 had the highest % porosity with the largest average pore diameter (Table 4) leading to greater peptide diffusion and hence, a faster release rate compared to other formulations. Whereas formulation F2, which exhibited the lowest porosity with the smallest average pore diameter (Table 4), showed the slowest release rate. Moreover, formulations F2 and F4, which have larger particle size and hence greater diffusion distance, exhibited slower release than formulations F1 and F3.

The RLD product, which is prepared using a gelatin based double emulsion method showed higher burst release than all the prepared formulations. This may be due to the smaller particle size (Fig. 2) of RLD compared to the prepared formulations leading to higher amount of surface associated drug. Moreover, the release profile of the RLD was tri-phasic, which may be a result of the presence of gelatin.

3.2.1. Peptide polymer interaction study

To further understand the impact of manufacturing differences on the drug release characteristics and the underlying release mechanism

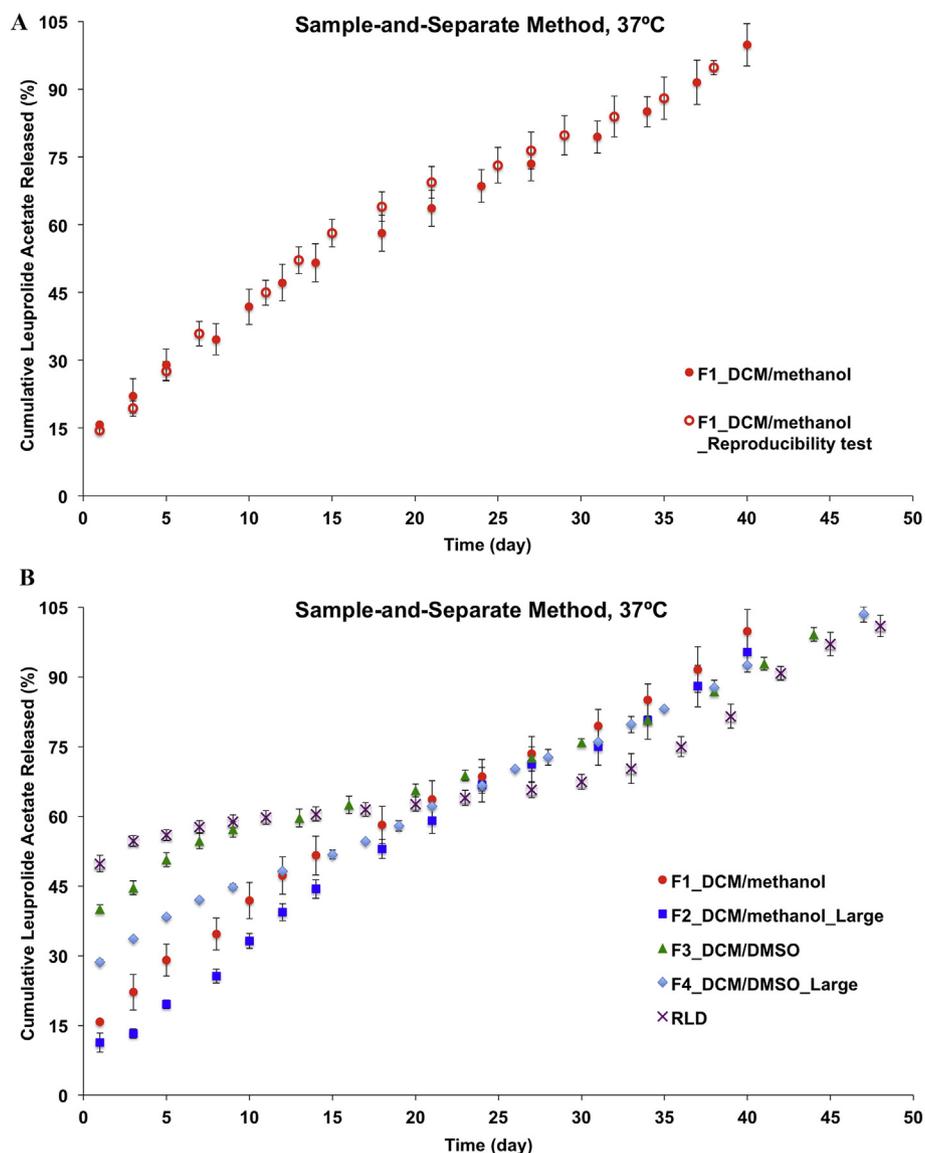


Fig. 4. A. *In vitro* release profiles of different batches of formulation F1 obtained on different testing dates using the sample-and-separate method at 37 °C ($n = 3$). The medium (33 mM PBS containing 0.02% w/w sodium azide and Tween 20, pH 7.4) was replaced every two to three days. B. *In vitro* release profiles of compositionally equivalent LA microspheres prepared with minor manufacturing changes and the RLD product obtained using the sample-and-separate method at 37 °C ($n = 3$). The medium (33 mM PBS containing 0.02% w/w sodium azide and Tween 20, pH 7.4) was replaced every two to three days.

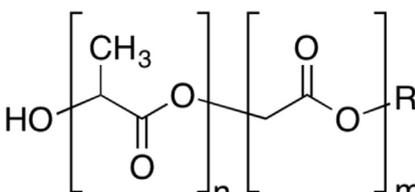
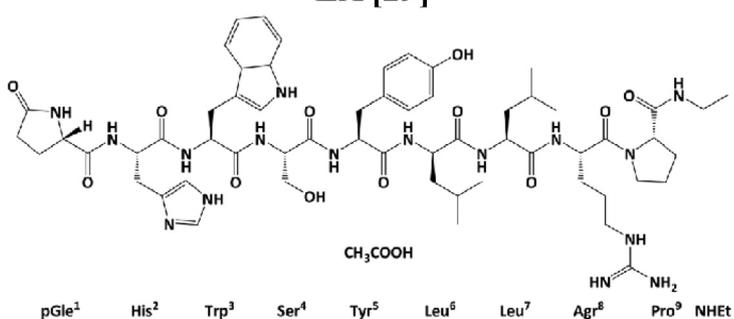
(s) of the prepared LA microspheres, peptide-polymer interactions as well as microsphere *in vitro* degradation were investigated.

It has been reported [25] that LA is a positively charged peptide at neutral or lower pH, with the potential to interact with the negatively charged carboxylic acid end groups in PLGA ($pK_a \sim 3.8$) [26]. This can result in the adsorption of LA within the microspheres PLGA matrix leading to slow release over a long period of time *via* desorption as the polymer degrades. Since this physicochemical interaction between LA and PLGA controls drug release [25], any unintended change to this interaction may result in undesirable changes in the drug release rate and duration. For example, solvents (DCM/DMSO/MeOH) have the potential to alter the physicochemical interaction between LA and PLGA during the manufacturing process. This in turn may alter peptide distribution as well as peptide-polymer interaction, resulting in alteration of the release characteristics.

FT-IR spectra of PLGA and LA in formulations F1 and F3 (prepared using different solvent systems) were evaluated for any changes (shift in the wavenumber or intensity) in their characteristic absorbance bands (Table 5) compared to pure LA, PLGA and their physical mixture

(Fig. 5). As shown in Fig. 5B, the intensity of the characteristic bands of LA in the physical mixture and the prepared LA microspheres were reduced compared to the pure LA spectra (Fig. 5A). This is due to the lower amount of LA (8%) compared to PLGA (92%). No other significant changes in the spectra of the microsphere formulations (Fig. 5B) were observed compared to the pure LA, PLGA and the physical mixture (Fig. 5A) except for the N–H stretching band. This indicates that there is no significant interaction between the drug and the polymer except for weak hydrogen bonding (N–H stretching band – 3269, 3323 and 3333 cm^{-1} in the physical mixture and formulations F1 and F3, respectively). Moreover, the FT-IR spectra of the formulations prepared with different solvent systems (Fig. 5B) were also similar except for the difference in the N–H stretching band. This indicates that there is no impact of the solvent system on LA-PLGA interaction in the dry microspheres, implying that the reported physicochemical interaction between LA and PLGA [25] possibly occurs only in the presence of aqueous media (water or release medium) at neutral or lower pH and not in the dry state.

Table 5
Characteristic FT-IR absorption bands (cm^{-1}).

Chemical structure	Characteristic absorption bands (cm^{-1})
PLGA [19,27,28]  LA [29] 	~2996, 2946 (CH, CH ₂ , CH ₃ stretching), ~1745 (ester C=O stretching), ~1381, 1423, 1451 (C=O bending vibrations), ~1268 (C-C(=O)-O symmetric/asymmetric stretching), 1150–1350 (CH ₂ , CH wagging), ~1082, 1128 (C–O ester stretching)
	~3269 – broad band (O–H, amide/amine N–H stretching), ~2996 (CH, CH ₂ , CH ₃ stretching), ~1630 (amide C=O stretching), ~1535, 1514 (N–H bending), ~1400–1600 (aromatic C=C stretching), ~1350–1480 (-CH (alkane) bending), ~1239 (C–N stretching)

3.2.2. *In vitro* degradation study

Hydrolytic degradation of the PLGA in aqueous release media is one of the major mechanisms for drug release from PLGA microspheres. It has been reported [15,16,30,31] that differences in the particle size and porosity of the prepared microspheres as a result of manufacturing differences may lead to an altered surface area to volume ratio and extent of water diffusion inside the particles and hence, differences in the polymer degradation rate. This may be responsible for the observed differences in the microsphere drug release characteristics. Therefore, an *in vitro* degradation study was conducted to evaluate any difference in the polymer degradation kinetics of the microspheres during release testing. However, as shown in Fig. 6, no significant differences in the polymer degradation rate (as shown by decrease in molecular weight)

were observed among the microspheres formulations with different size and porosity. This may be a result of the faster degradation rate of the low M.wt. PLGA of LA microspheres compared to that of previously reported high molecular weight PLGA microspheres [32]. Thus, the differences in release characteristics of the various microsphere formulations are not due to the changes in polymer degradation rate.

The diffusion of the peptide from the polymer matrix into the release media is another major release mechanism. Microsphere samples from the *in vitro* degradation study were evaluated for impact of morphological differences (such as pore diameter, shape and network) on the diffusion-based peptide release. As shown in Fig. 7, as degradation proceeded, both microsphere formulations appeared to follow the typical “inside-out” degradation mechanism. It was evident that the more

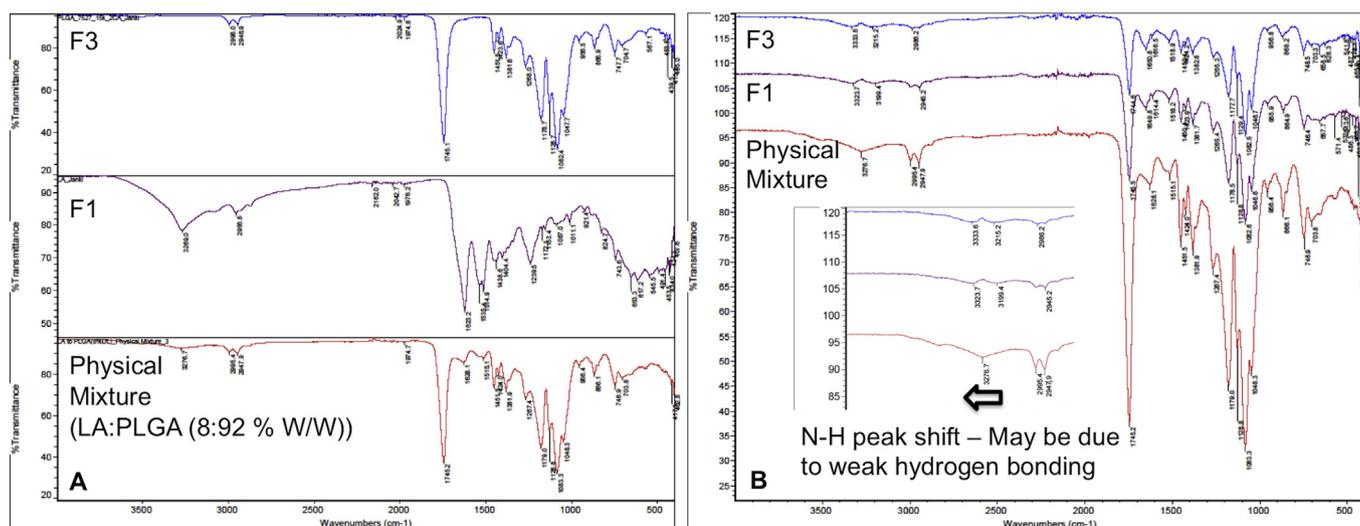


Fig. 5. (A) Overlay of FT-IR spectra of LA, PLGA and physical mixture (LA:PLGA, 8:92% w/w) and (B) Overlay of FT-IR spectra of prepared LA microsphere formulations F1_DCM/MeOH and F3_DCM/DMSO and physical mixture (LA:PLGA, 8:92% w/w).

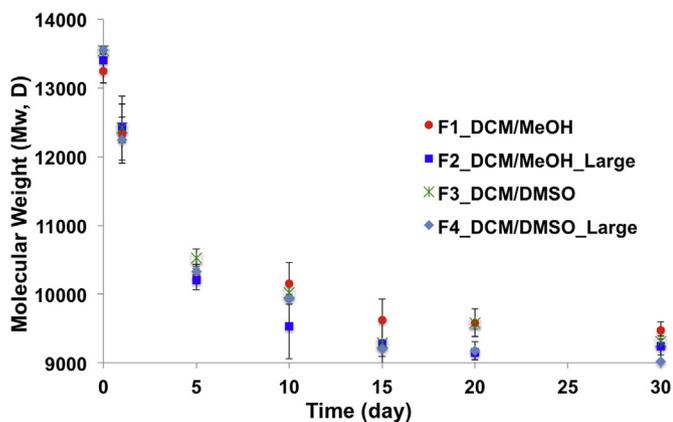


Fig. 6. Degradation kinetics of PLGA microspheres prepared with minor manufacturing differences under *in vitro* release testing conditions (33 mM PBS containing 0.02% w/w sodium azide and Tween 20, pH 7.4 at 37 °C).

porous microspheres *i.e.* formulation F3 degraded faster with formation of large pores and channels inside the microspheres by day 15 (as shown by the yellow arrow) compared with the less porous microspheres (*i.e.* formulation F1). The formation of pores and channels inside the F1 formulation became evident only after day 15. These differences in microsphere structure may be responsible for the observed differences in the drug release rates during the initial 15-day period (Fig. 4B), especially since LA is large in size and hence, may have pore size/structure restricted diffusion and release. After 15 days, polymer erosion progressed extensively leading to complete shape deformation and exposure of greater surface area. No significant difference in pore structure and hence, release rate was observed after day 20 (Fig. 4B).

Similar morphological changes as formulations F1 and F3 were observed for formulations F2 and F4, prepared using the same solvent system despite their larger size (data not shown).

3.3. *In vivo* release characteristics of the LA microspheres

The pharmacokinetic profiles of the LA microspheres following intramuscular (*i.m.*) administration in rabbits are shown in Fig. 8. All the prepared compositionally equivalent LA microsphere formulations and

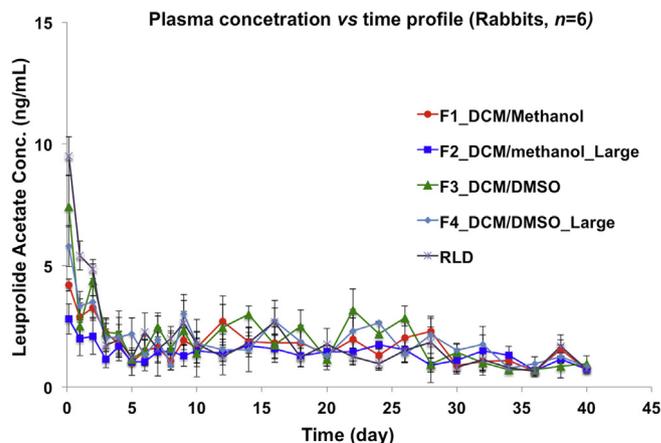


Fig. 8. *In vivo* release profiles of the compositionally equivalent LA microspheres prepared with minor manufacturing changes in a rabbit model (*i.m.* n = 6).

Table 6
Pharmacokinetic parameters of leuprolide acetate microspheres (*i.m.*, rabbits).

Formulation (n = 6)	T _{max} (day)	C _{max} (ng/mL)	AUC _{0-last} (µg/L * day)	T _{1/2} (day)
F1	0.15	4.22	66.97	17.73
F2	0.15	2.87	54.89	23.83
F3	0.15	7.43	76.30	15.59
F4	0.15	5.80	74.69	12.42
Lupron Depot®	0.15	9.49	69.03	24.15

the RLD product exhibited bi-phasic *in vivo* release profiles with variable burst release phase followed by slow and continuous zero-order release over 40 days. The key pharmacokinetic parameters of each microsphere formulation were determined and are listed in Table 6. Similar T_{max} of 3.5 h with variable C_{max} concentrations corresponding to the burst release were observed for all formulations and the RLD product (Table 6). Formulation F2 had the lowest C_{max} while formulation F3 had the highest C_{max} with F1 and F4 having intermediate C_{max}. Moreover, formulation F2 had the lowest AUC_{0-last} while F3 had the highest AUC_{0-last} with F1 and F4 having intermediate AUC_{0-last}

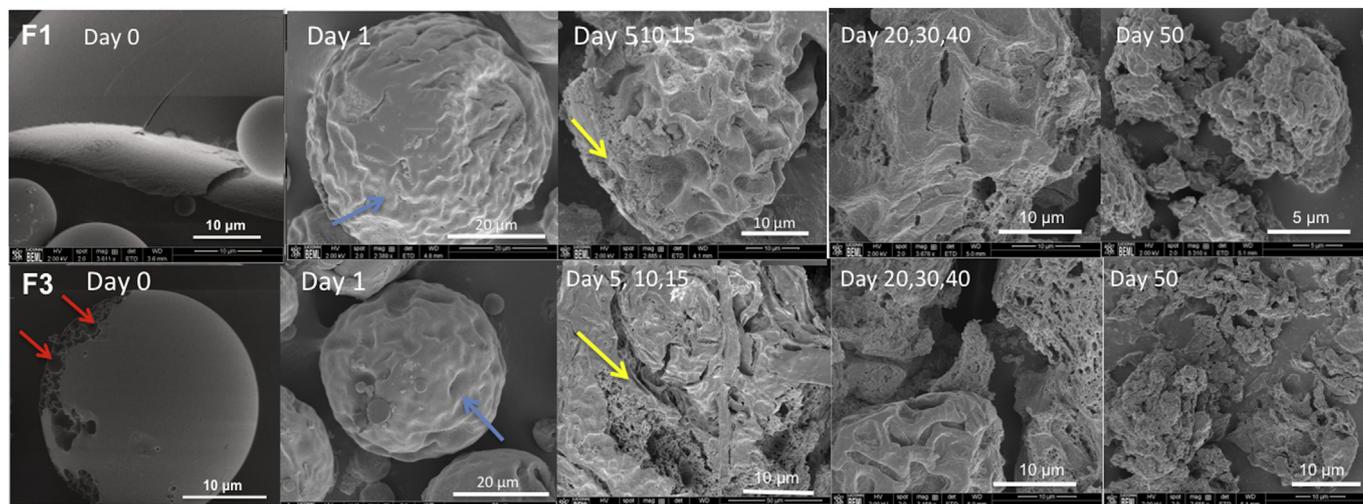


Fig. 7. SEM images of LA microsphere formulations F1_DCM/MeOH and F3_DCM/DMSO under *in vitro* release testing condition (33 mM PBS containing 0.02% w/w sodium azide and Tween 20, pH 7.4 at 37 °C) over a period of 50 days. Symbols: the red arrows point to pores on the surface of the microspheres; the blue arrows point to differences in the indentations on the surface of the microspheres; the yellow arrows point to the differences in pore structure. Due to the similar morphological changes at day 5, 10 and 15, only one representative image is shown for each formulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 7

Pharmacokinetic parameters of the LA solution (*i.v.*) (Software: WinNonlin® 6.4).

A	B	α (h^{-1})	β (h^{-1})	K_{10} (h^{-1})	K_{12} (h^{-1})	K_{21} (h^{-1})
46.34	6.40	4.90	0.39	2.04	2.31	0.93

values. F2 had the longest $T_{1/2}$ compared to all other formulations.

The differences in the *in vivo* burst release and release rate are similar to the *in vitro* release profiles of the various formulations and result from differences in their physicochemical properties.

3.4. Pharmacokinetic analysis and development of IVIVC

3.4.1. Pharmacokinetic analysis of LA in rabbits

The PK study of the LA solution following single intravenous (*i.v.*) administration was used to determine its PK parameters (Table 7) *i.e.* the distributive and elimination micro rate constants (k_{12} , k_{21} and k_{10}), which are necessary for deconvolution of the LA microsphere *in vivo* release profiles into the total fraction absorbed using the Loo-Riegelman method [20].

3.4.2. Deconvolution of the *in vivo* release profiles of LA microspheres

The *in vivo* plasma concentration vs time (PK) profiles (*i.m.*) of the LA microspheres were deconvoluted into the fraction absorbed vs time using the pharmacokinetic parameters (Table 7) of LA in rabbits and the Loo-Riegelman deconvolution method [20]. The deconvoluted fraction absorbed vs time profiles of the prepared LA microspheres and the RLD product are shown in Fig. 9. Overall, the deconvoluted *in vivo* LA release from PLGA microspheres appeared to be faster compared to their respective *in vitro* release profiles. This may be due to accelerated polymer degradation as a result of local acidic pH and/or the presence of other biological components (such as enzymes) in the interstitial tissue fluid at the intramuscular injection site. Despite the difference in the *in vivo* and *in vitro* release rates, the *in vivo* release profiles of all formulations followed a similar rank order as the *in vitro* release profiles: RLD > formulation F3 > formulation F4 > formulation F1 > formulation F2. This indicates that the release mechanisms under *in vitro* and *in vivo* conditions appear to be similar, which is one of the major requirements for the development of IVIVCs [1]. Interestingly, it was observed that the *in vivo* burst release was significantly lower than each respective *in vitro* burst release. This may be due to the masking effect of poor or slow absorption of the peptide into the systemic circulation as a result of hydrophilicity, larger size, steric hindrance and/or charge interaction

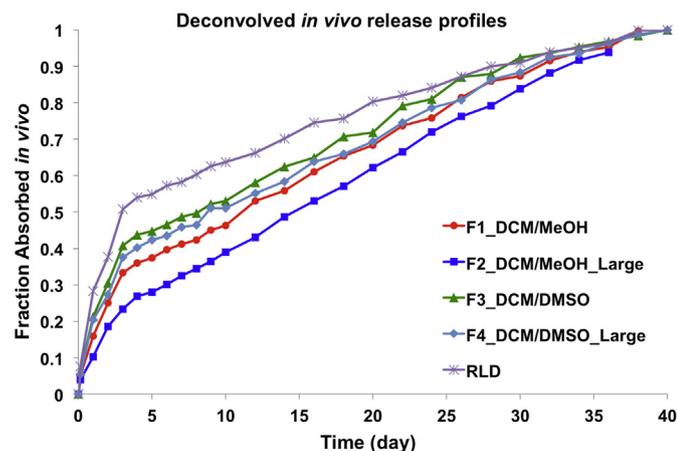


Fig. 9. Deconvoluted *in vivo* profiles (fraction absorbed/released) of the compositionally equivalent LA microspheres prepared with minor manufacturing changes (deconvoluted using the Loo-Riegelman method).

with the extracellular matrix at the intramuscular site.

Overall, comparison of the deconvoluted *in vivo* release profiles with the respective *in vitro* release profiles of the LA microspheres suggested the feasibility of developing a point-to-point IVIVC model, but with the use of mathematical parameters to account for the difference in the release rate and % burst release under *in vitro* and *in vivo* conditions.

3.4.3. *In vitro* - *in vivo* correlation of the LA microspheres

The U.S. FDA guideline [1] recommends the use of a minimum of two, preferably three or more formulations with different release rates to define an IVIVC. Therefore, all four prepared LA microsphere formulations with different *in vitro* and *in vivo* release rates were used in developing IVIVC(s) in different combinations of three out of four formulations. Based on the knowledge obtained from our previous research on risperidone [15] and naltrexone [16] microspheres, formulations on the extremes of the release profile range (*i.e.* formulations F2 and F3) were included in the different combinations of formulations studied to develop correlation models with better predictability and wider application scope. Accordingly, a total of two different combinations of three formulations (*i.e.* F1, F2, F3 and F2, F3, F4) were investigated. First of all, the *in vitro* release profiles were simulated *via* model fitting using Origin Pro (data analysis software) to synchronize the sampling time interval with the *in vivo* release profiles. Due to the differences in the release rate and release duration of the *in vitro* and deconvoluted *in vivo* release profiles, time scaling (1/1.65) and time shifting (+1) factors (determined using a Levy plot - *in vivo* vs *in vitro* time for definite fraction released) were used to achieve a 1:1 correlation. As recommended by the FDA guidance, the same time-scaling/shifting factors were applied to all formulations used for the development and validation of the IVIVCs. The fraction absorbed/released *in vivo* of selected formulations was plotted against the fraction released *in vitro* at the respective time point to establish a correlation if any. As shown in Fig. 10 (A and D), an affirmative point-to-point correlation *i.e.* a Level A IVIVC [1] between the fractions released *in vitro* and fractions released/absorbed *in vivo* was observed for all combinations using a simple linear regression model (correlation coefficients > 0.97). All the developed IVIVCs were comparable as manifested by similar slopes and intercepts.

These developed IVIVCs were then used to predict the *in vivo* profiles of the fourth remaining external formulation using the real time *in vitro* release profiles. As shown in Fig. 10 (B and E), the predicted *in vivo* profiles of the external formulations overlapped with the observed deconvoluted *in vivo* release profiles for both IVIVC models, indicating that the models are predictable.

The developed IVIVCs were also used to predict the *in vivo* release/absorption profile of the Lupron Depot®, RLD product from its “real-time” *in vitro* release profile. As shown in Fig. 10 (C and F), the predicted *in vivo* release profiles were very similar to the observed deconvoluted *in vivo* profiles obtained in rabbits irrespective of which IVIVC model was used. These results showed that the developed IVIVCs were sufficiently robust. Most importantly, the developed IVIVCs can be used to predict not only the LA microsphere formulations that are equivalent in formulation composition but also a microsphere formulation that is not equivalent in composition but has similar drug loading.

All the developed IVIVCs were internally as well as externally validated for their predictability as per the FDA guidance [1]. The predictability of the IVIVCs was estimated in terms of % prediction error (PE) of C_{max} and $\text{AUC}_{0-\text{last}}$ using WinNonlin® 6.4 software. As shown in Table 8, the average absolute internal % PE for the C_{max} (9.52%) of IVIVC 2 was within the recommended range of 10% or less. However, the average absolute internal % PE for $\text{AUC}_{0-\text{last}}$ (10.94%) was slightly > 10%, suggesting the internal predictability of the developed IVIVC 2 for the $\text{AUC}_{0-\text{last}}$ was inconclusive. Accordingly, evaluation of external predictability of the IVIVC 2 was performed. As can be seen in Table 8 the external % PE for the C_{max} and $\text{AUC}_{0-\text{last}}$ were 1.52% and

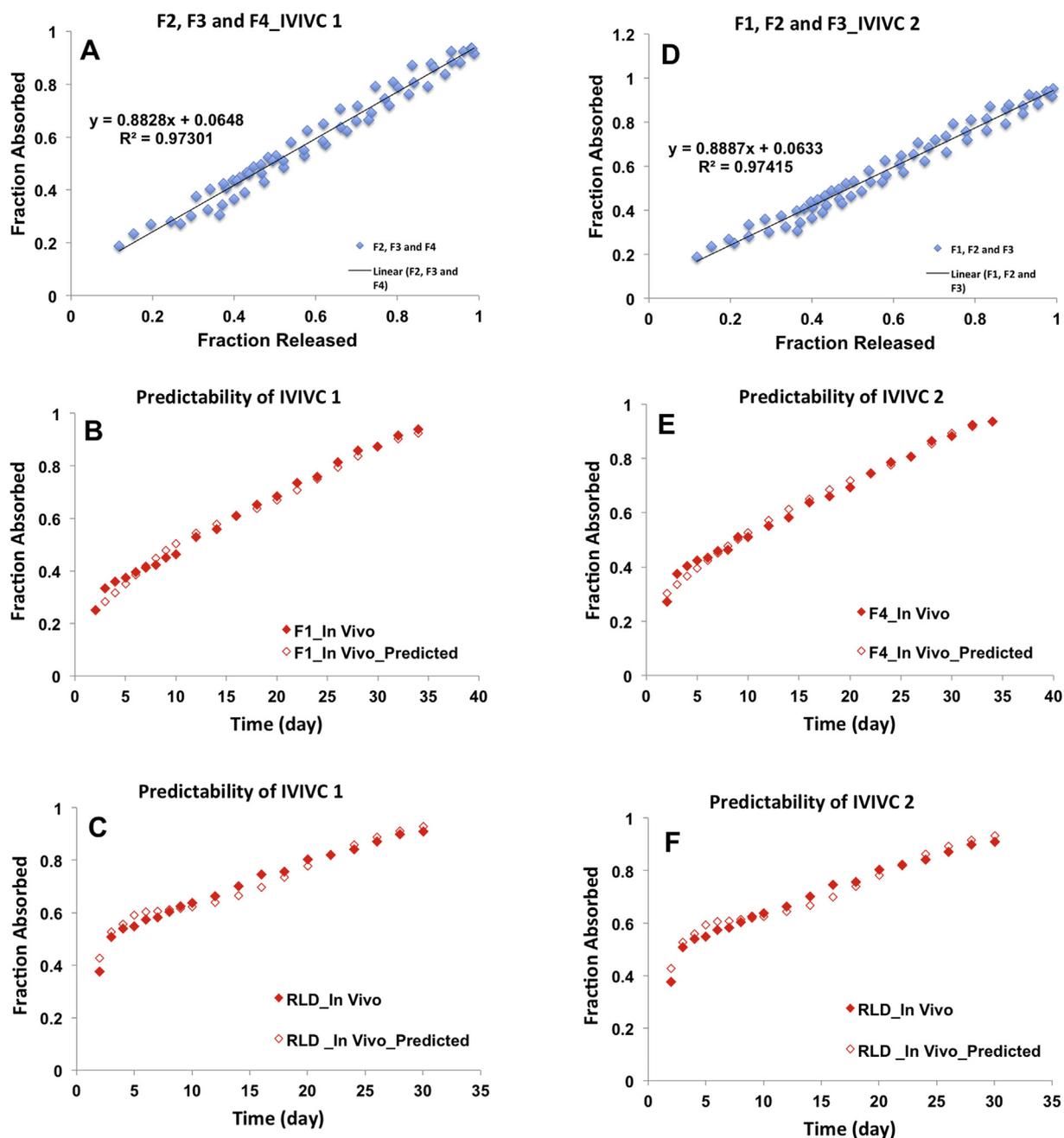


Fig. 10. Level A IVIVC for LA microspheres using the two-stage deconvolution approach based on the Loo-Reigelman method. (A) IVIVC developed using formulations F2, F3 and F4 (IVIVC 1). (B) Experimental and predicted *in vivo* release profiles of formulation F1, based on IVIVC 1. (C) Experimental and predicted *in vivo* release profiles of RLD product, Lupron Depot®, based on IVIVC 1. (D) IVIVC developed using formulations F1, F2 and F3. (E) Experimental and predicted *in vivo* release profiles of formulation F4, based on IVIVC 2. (F) Experimental and predicted *in vivo* release profiles of RLD product, Lupron Depot®, based on IVIVC 2.

2.83%, respectively, which passes the recommended external predictability evaluation (% PE of 10% or less). These results indicate that the developed IVIVC 2 has good external predictability, and thus could be used as a surrogate for bioequivalence studies in rabbits.

Furthermore, the predictability of the developed IVIVC 2 for the RLD was also investigated. Both % PEs for C_{max} (8.40%) and AUC_{0-last} (–8.31%) were below 10%. These results confirmed that the developed IVIVC 2 has potential to be used to predict the *in vivo* performance of not only the compositionally equivalent LA microsphere formulations with manufacturing differences but also LA microsphere formulations that are not compositionally equivalent. Moreover, the successful development of the validated IVIVC 2 model also indicates that the developed sample-and-separate *in vitro* release testing method had good

predictability of the *in vivo* performance of leuprolide acetate polymeric microspheres.

Please note that external % PE for the IVIVC_1 was within the acceptance criteria in terms of C_{max} (–8.92%) but slightly higher for the AUC_{0-last} (–10.85%). Accordingly, it was not considered a validated IVIVC model although it did not show significant difference in the predictability of *in vivo* profile (Fig. 10 B and C).

4. Conclusions

The present manuscript describes for the first time that Level A IVIVCs can be developed for compositionally equivalent PLGA microspheres containing large molecular weight and complex molecules such

Table 8
Validation and prediction of the developed IVIVC₂ for LA microspheres.

	C _{max} (µg/L)			AUC _{0-last} (µg/L * day)		
	Obs.	Pred.	%PE	Obs.	Pred.	%PE
Internal validation						
Formulation F1	4.22	3.96	-6.18	66.97	61.42	-8.28
Formulation F2	2.87	2.38	-16.95	54.89	62.68	14.20
Formulation F3	7.43	7.02	-5.43	76.30	68.41	-10.34
Average Internal	4.84	4.45	9.52	66.05	64.17	10.94
External validation						
Formulation F4	5.80	5.71	-1.52	74.69	72.58	-2.83
RLD prediction						
Lupron Depot®	9.49	10.29	8.40	69.03	63.29	-8.31

as peptides. The IVIVC developed was also predictive of the RLD product, Lupron Depot® in a rabbit model. It was observed for the first time that slow absorption of peptide at the intramuscular site masked the high burst release that was observed under *in vitro* release testing conditions. This difference in the burst release phase under *in vitro* and *in vivo* conditions complicated the development of an IVIVC. Accordingly, multiple mathematical parameters were needed to achieve a 1:1 linear Level A correlation. Together with our previous research on developing Level A IVIVCs for polymeric microspheres containing small molecules, it can be concluded that Level A IVIVCs can be developed for a variety of complex parenteral microsphere drug products using *in vitro* release testing methods and a rabbit model. This shows the potential of developing IVIVCs using clinical data for such complex parenteral drug products, which can be used in product development and regulatory decision making and shows the potential for such IVIVCs to serve as surrogates for bioequivalence studies.

The critical quality attributes and performance of microspheres containing large molecular weight and complex molecules such as peptides were shown to be sensitive to minor manufacturing changes. This is particularly significant for microspheres containing substantial amount of potent drugs such as peptide. It was also shown that the hydrolytic degradation of low molecular weight PLGA was not sensitive to differences in particle size and porosity. However, the drug release characteristics of such microspheres containing relatively large hydrophilic molecules such as peptide were sensitive to changes in the microsphere pore structure.

Disclaimer

The views expressed in this abstract do not reflect the official policies of the U.S. Food and Drug Administration or the U.S. Department of Health and Human Services.

Acknowledgements

This work was financially supported by the Office of Research and Standards, Office of Generic Drugs, CDER at the FDA (1U01FD004931-02).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2019.07.013>.

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